AD_____

Award Number: DAMD17-99-1-9440

TITLE: Cell Surface Regulation of Matrix Metalloproteinases in Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Rafael A. Fridman, Ph.D.

CONTRACTING ORGANIZATION: Wayne State University
Detroit, Michigan 48202

REPORT DATE: August 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2001	Annual (1 Jul 00 - 1 Jul 01)

4. TITLE AND SUBTITLE
Cell Surface Regulation of Matrix Metalloproteinases in Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-99-1-9440

6. AUTHOR(S)
Rafael A. Fridman, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Wayne State University
Detroit, Michigan 48202
E-Mail:

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Metastasis is the major cause of death in breast cancer patients and is partly caused by the action of proteolytic enzymes that degrade extracellular matrix (ECM). We have focused on the gelatinases, MMP-2 and MMP-9, two ECM-degrading enzymes that are members of the matrix metalloproteinase (MMP) family of proteases. The gelatinases are associated with the surface of breast cancer cells. MMP-2 surface binding plays a role in activation by MT1-MMP, a membrane-bound MMP that is also expressed in breast cancer. MMP-2 activation is mediated by the action of TIMP-2, a metalloproteinase inhibitor. However, the role of TIMP-2 in pro-MMP-2 activation by MT1-MMP is not clear. Although MMP-9 is identified on the cell surface, the mechanism of MMP-9 surface association and regulation remains to be investigated. The research of the Academic Award during the second year has focused on the mechanisms involved in pro-MMP-2 activation and MMP-9 surface association. We report a detailed characterization of the activation of pro-MMP-2 and the interactions of TIMP-2 and TIMP-4 with MT1-MMP during this process. We also carried phage display studies to identify MMP-9-binding proteins.

14. Subject Terms	15. NUMBER OF PAGES
metalloproteinases, protease inhibitors, breast cancer, metastasis	34
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Background. The matrix metalloproteinase (MMP) family of endopeptidases has been shown to play a central role in the promotion of tumor invasion and angiogenesis in breast carcinomas (1, 2) by their ability to mediate the degradation of extracellular matrix (ECM) components as well as non-ECM proteins. A key aspect of proteolytic degradation in cancer cells involves the targeting of enzymes to the cell surface. Studies with various protease systems have shown that surface association of ECM-degrading proteases in cancer cells regulates enzyme activity and interactions with protease inhibitors (3, 4). To achieve their full potential in pericellular proteolysis, the members of the MMP family evolved into secreted and membrane-tethered multidomain enzymes by incorporating distinct domains that facilitate binding to ECM components and surface molecules in the case of soluble MMPs such as the gelatinases (MMP-2 and MMP-9) and unique domains that anchors the enzyme to the cell surface, in the case of the MT-MMPs.

The gelatinases (MMP-2 and MMP-9) represent a subgroup of MMPs that can degrade basement membrane collagen IV and therefore they have been implicated in metastasis in many human tumors including breast cancer (1). As soluble MMPs, the gelatinases developed unique mechanism of surface association. For example, the latent form of pro-MMP-2 binds to a surface complex of MT1-MMP with TIMP-2, which plays a role in activation of pro-MMP-2 (5). Pro-MMP-9 binds to the cell surface via the α2(IV) chain of collagen IV or via the hyaluronic receptor CD44 (3). However, their roles in MMP-9 regulation are not known. Furthermore, other surface-binding proteins may exist.

MT1-MMP is a member of the MT-MMP subfamily of MMPs that is anchored to the cell surface by the presence of a transmembrane domain (6). MT1-MMP is expressed in both breast cancer cells and in the tumor stroma (7). As membrane-tethered MMP, MT1-MMP plays a key role in pericellular proteolysis and is a known activator of pro-MMP-2 on the cell surface. The activation process involves the action of TIMP-2, which binds to active MT1-MMP forming a surface "receptor" for pro-MMP-2. The interactions of TIMP-2 with MT1-MMP and the role they play in pro-MMP-2 activation and MT1-MMP regulation are not well understood. TIMP-4, another member of the TIMP family, can form a non-covalent complex with pro-MMP-2. However its role in pro-MMP-2 activation by MT1-MMP is unknown.

Purpose. The purpose and scope of the Academic Award proposal is to focus our research efforts in understanding the cell surface regulation of the gelatinases, MMP-2 and MMP-9, and MT1-MMP with the goal to decipher the function and role of these enzymes in breast cancer progression.

BODY

Note: Since the results of the following section have been published, we respectfully request the reviewers to refer to the enclosed publications for details in experimental procedures and results.

1. Studies on MT1-MMP and MMP-2 Regulation. **(J. Biol. Chem., 275, 41415-41423, 2000).**

As a continuation of our studies aimed to understand the regulatory effects of MT1-MMP inhibition in pro-MMP-2 activation, we carried out studies with synthetic MMP inhibitors (MMPI). We previously showed that pro-MMP-2 activation by MT1-MMP was a process dependent on TIMP-2 (8). Studies on the processing of MT1-MMP revealed an unexpected effect of TIMP-2. In the presence of the inhibitor, the cells accumulated the active form of MT1-MMP on the cell surface due to a direct inhibition of autocatalytic processing (8). We hypothesized that accumulation of active MT1-MMP, as a consequence of decreased enzyme turnover, would increase the number of MT1-MMP molecules available on the cell surface. In the presence of TIMP-2 this would generate additional MT1-MMP/TIMP-2 complexes and therefore enhance pro-MMP-2 activation. Hence, inhibition of autocatalytic processing would "feed" MT1-MMP molecules into the ternary complex system further enhancing pro-MMP-2 activation. This suggested the hypothesis that TIMP-2 enhancement of pro-MMP-2 activation by MT1-MMP involves both formation of ternary complex and inhibition of MT1-MMP processing. To test the importance of inhibition of MT1-MMP autocatalytic processing, we used synthetic MMPIs such as Marimastat. This inhibitor also binds MT1-MMP and induces accumulation of the active form of MT1-MMP. We showed that MMPIs could act synergistically with TIMP-2 in the promotion of pro-MMP-2 activation by MT1-MMP. In contrast, TIMP-4, an efficient MT1-MMP inhibitor, had no synergistic effect. These studies suggest that under certain conditions the pericellular activity of MT1-MMP in the presence of TIMP-2 can be modulated by synthetic and natural (TIMP-4) MMP inhibitors. This study has been published, as shown above.

We also found that the ectodomain of MT1-MMP is shed into the extracellular space, while in the membrane remains a 44-kDa inactive species. This process occurs at low TIMP-2 concentrations and is consistent with the autocatalytic nature of the MT1-MMP processing. While this process would terminate activity on the plasma membrane, the shed ectodomain contains the catalytic domain and if functional may contribute to pericellular proteolysis. The characterization of this process is ongoing.

2. Studies on TIMP-4 and MT1-MMP. **(Bioch. Biophys. Res. Comm., 281, 126-130, 2001).**

The tissue inhibitors of metalloproteinases (TIMPs) are specific inhibitors of MMP enzymatic activity. However, TIMP-2 can promote the activation of pro-MMP-2 by MT1-MMP. This process is mediated by the formation of a complex between MT1-MMP, TIMP-2 and pro-MMP-2. Binding of TIMP-2 to active MT1-MMP also inhibits the autocatalytic turnover of MT1-MMP on the cell surface. Thus, under certain conditions, TIMP-2 is a positive regulator of MMP activity. TIMP-4, a close homologue of TIMP-2 also binds to pro-MMP-2 and can potentially participate in pro-MMP-2 activation. We co-expressed MT1-MMP with TIMP-4 and investigated its ability to support pro-MMP-2 activation. TIMP-4, unlike TIMP-2, does not promote pro-MMP-2 activation by MT1-MMP. However, TIMP-4 binds to MT1-MMP inhibiting its autocatalytic processing. When co-expressed with TIMP-2, TIMP-4 competitively reduced pro-MMP-2 activation by MT1-MMP. A balance between TIMP-2 and TIMP-4 may be a critical factor in determining the degradative potential of cells in normal and pathological conditions.

3. Studies on MMP-9.

Identification of pro-MMP-9 Interacting Proteins. Since pro-MMP-9 binds to the surface of breast cancer cells, we set up to identify novel proteins that may interact with pro-MMP-9. In our original application we proposed to utilize a biochemical approach using immobilized MMP-9 and cell extracts. Instead, we decided to try a genetic approach using phage display. We thought that this system would allow us to identify interacting proteins and obtain their gene sequence directly. To this end, we carried out studies on affinity screening of a T7 Phage displayed breast tumor cDNA library. The tumor used for making this random primed Human cDNA library (Novagen, WI) was from a 46-year old woman suffering from moderately differentiated invasive ductal carcinoma. The number of primary clones in the library was 1.6×10^7. The recombinant human pro-MMP-9 used for biopanning was the 92-kDa latent form produced in the lab using our vaccinia expression system in mammalian cells (9). Recombinant pro-MMP-9 was purified to homogeneity as described (10). Trace amounts of TIMP-1 in the recombinant pro-MMP-9 preparation were removed by Heparin-agarose affinity. During the second year (00-01), we carried out the screening assay as follows:

Protocol:

- Each well of ELISA plate was coated with 1μg of Pro MMP-9. Control wells were coated with BSA alone.
- The non-specific binding sites were blocked with 5% protease free BSA

- 3.4×10^9 pfu of the amplified phage display library was added in each well for affinity interaction between phage expressed proteins and Pro-MMP9. The plates were incubated for 1hr. at 37°C to pick up high affinity clones.

- The non-binders were washed away with TBST. The stringency of washing increased with each round of screening.

- The bound phage was eluted with 0.1%SDS. Eluted phage was amplified on BLT5615 cells before each round of screening.

- The eluted bound phages were plated out at log dilutions 10^3, 10^4 and 10^5 in duplicate before amplification after each round of screening to determine the enrichment of the selected clones after each round of screening.

- After the final round of screening 170 plaques were picked up from the plates, dispersed in extraction buffer, mixed with equal volume of EDTA and heated at 65°C for 10'.

- For each of the 170 clones a PCR reaction was set up using T7 UP and DOWN primers derived from the flanking regions of the phage coat protein.

- The PCR products were purified using standard molecular biology procedures and run on 2% agarose gel.

- The PCR products were grouped in 10 sets based on their size and digested with Alu1 and run on a 2% gel to check for similar clones.

- One clone from each of the similar clone sets was sequenced using T7 UP and DOWN primers.

- From the sequences obtained, NCBI BLAST was performed using NR and SWISS PROT databases to search for homology.

Results:

Starting from a titer of 2.12×10^4 of the clones from wells coated with pro-MMP-9 alone almost a 100 fold enrichment of the plaques (3.76×10^6) was obtained after the 7^{th} round of screening. The plaques binding non-specifically to control wells coated with BSA alone were lost completely with increased stringency of washing after the 3^{rd} round of screening (data not shown). The selected clones were grouped in 9 sets based on their sizes ranging down from 1000 bp to 350 bps and an Alu1 digestion was performed to search for similar and different clones (Table 1). Out of 170 clones picked 59 clones were about 600 bp in size and represented the most selected clone. However, when an Alu 1 digestion was performed two patterns of digestion were observed showing that the class of clones representing 600bp actually comprised of two different sets of clones. The clones representing sizes between 350

and 375 bp turned out to be the same upon sequencing representing 19% (4+15) of the total selected clones. The next in abundance were the ~1000bp clones (18 in number) constituting 10 % of the total. The remaining clones did not show a defined digestion pattern and thus were not further investigated.

Table 1: Distribution of clones after digestion with AluI

Clone class on the basis of size	# of clones	% of total
1000	18	~10%
900	1	~0.5%
800	5	~3%
620	30	~18%
607	29	~17%
500	3	~2%
410	2	~1%
390	7	~4%
375*	6	~4%
350*	26	~15%
Total	127	~ 75%

* Same translated sequence 36 amino acids from the point of fusion

Blastp, Blastn and tblastx were used to find significant homologies at nucleotide and protein levels. Blastp compares an amino acid query sequence against a protein sequence database. Blastn compares a nucleotide query sequence against a nucleotide sequence database. Tblastx compares six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence.

Blastp results:

The nucleotide sequence downstream of the phage coat protein and in alignment with its reading frame was translated and the sequence (only the sequence before the first stop codon was considered) of the amino acids obtained was used in **blastp** of NCBI NR database and the Swissprot database. The results of the blast have been summarized in Tables 2, 3, 4, 5, 6 and 7. The Bit score value 'S' is derived from the raw alignment score in which the statistical properties of the scoring system used have been taken into account. These scores have been normalized with respect to the scoring system; they can be used to compare alignment scores from different searches. 'E' is the Expectation value and represents the chance occurrence of an alignment during the search. The lower the E-value, the more significant is the score. Homologies with E-values greater than 100 were restricted out from the blast search.

Table 2: blastp of the 1000bp clones

Clone size	# of clones	% of total	# of amino acids from point of fusion	
~1000 bp	18	11%	44	
NR Database			S	E
Unnamed Protein product (AK022872) (codes for 443 aa)			24	36
Hypothetical proteinFLJ12810			24	36
Ig lambda chain precursors			23-22	55-85
Swiss Prot				
FHR4 Factor H-Related Protein 4 precursor			21	31
Ig lambda chain C regions			21	40
Microtubule-associated protein 1B			20	94
Integrin β6-precursor			20	96

Table 3: blastp of a subset of 600 bp clones

Clone size	# of clones	% of total	# of amino acids from point of fusion	
~600 bp	29	17.05%	51	
			S	E
NR Database				
Hypothetical Protein KIAA0559			25	7.0
Hypothetical protein FLJ13725			25	9.2
Intercellular adhesion molecule 5 precursor (ICAM)			24	18
Immunoglobulin heavy chain variable region			24	21
Lung Cancer Antigen NY-LU-12			23	48
Melanoma Antigen MAGE A9			23	51
Chondroitin sulfate proteoglycan 2 (VERSICAN)			23	53
Swiss Prot				
Myosin IF			23	11
Melanoma Associated Antigen MAGE-9			22	18
Versican Core protein Precursor			22	20
Corticotropin releasing Factor Receptor 1 precursor (CRF-R)			22	23
Restin (Cytoplasmic linker protein-170 alpha-2) (CLIP170)			21	39
IL-2 Receptor β-Chain			21	40
Integrin α-M precursor (Cell Surface Glycoprotein MAC-1 α-subunit) (CR-3 α chain) (CD11B) Leukocyte adhesion receptor MO1) (neutrophil adherence receptor) Integrin α-X precursor Leukocyte adhesion glycoproteinP150, 95 α-chain (CD11c) (leu M5) Integrin α-D precursor (leukointegrin α-D) (CD11d)			20	63
Adapter Related protein Complex 2 α1 subunit (clathrin assembly protein complex 2)			20	64
MAGE-2 antigen			20	67
MAGE-12 antigen			20	85
Cell adhesion kinase (tyrosine-protein kinase CAK)			20	91
(Epithelial discoidin domain receptor 1 precursor)			20	91
Insulin-like growth factor I receptor precursor				

Table 4: blastp of 350bp, 500bp and 800bp clones

Clone sizes	# of clones	% of total	# of amino acids from point of fusion
375 bp	6	3.5%	36
350 bp	26	15%	36
500 bp	3	1.76%	15
800 bp	5	2.94%	23

	S	E
350 and 375 bp clones		
Transmembrane 7 superfamily member	24	30
Brain tumor associated protein NAG14	23	56
Hypothetical protein FLJ20772	23	74
KIAA0298 protein	23	78
VWF factor precursor (Coagulation factor VIII)	22	94
500 bp clone		
Dipeptidyl Peptidase IV like protein	20	82
800 bp clone		
<u>NR Database</u>		
55-C-FOS Protooncogene	22	29
Hypothetical protein KIAA00..	22	37
SACSIN(may function in chaperone mediated folding)	21	51
Protein Kinase CLK3	21	56
Sorting Nexin13	21	72
BRC2 (breast Cancer type2 susceptibility protein)	21	84
Brain-specific angiogenesis inhibitor 2 precursor	21	85
<u>SwissProt</u>		
Sacsin	21	36
Contactin precursor (glycoprotein GP135) (contains 6Ig-like C2-type domain and 4 fibronectin typeIII-like domains)	20	74
Dipeptidyl Peptidase IV like protein-Dipeptidyl aminopeptidase related protein	20	82

Table 5: blastp of the 900bp clone

Clone size	% of clones	% of total	% of amino acids from point of fusion	
900 bp	1	0.5%	91	
NR Database			S	E
Opioid growth factor receptor			28	3.4
Suppression of tumorigenicity 5			27	7
KIAA0672 gene product			26	12
C-KIT proto-oncogene (Proto-oncogene tyrosine protein kinase)			26	17
Lectomedin-2 (KIAA0821 protein) (a family of heptahelical receptors with adhesion-like domains)			26	22
Hypothetical protein FLJ20753			25	36
Phospholipase C			24	42
Hypothetical and unnamed proteins			25-23	29-92
Swiss Prot				
Mast/Stem Cell growth factor receptor precursor (SCFR) C-KIT, CD117 antigen			26	3.2
Phospholipase C-β-3			24	7.7
Basement Membrane Specific heparan Sulfate Proteoglycan core (PERLECAN)			23	21
Macrophage Colony Stimulating Factor I Receptor precursor (CD115)			23	23
Low Affinity immunoglobulin gamma Fc region receptor II B precursor (CD32)			23	24
Alpha adducing			23	24
Coagulation factor X precursor (Stuart Factor)			23	39
MAGE 12			22	44
MAGE-2			22	44
IG Lambda Chain V-II region			22	49
VEGFR-2 precursor			21	70

Table 6: blastp of the 400bp clones

Clone size	% of clones	% of total	Number of base pairs	% of amino acids from point of fusion	
~400 bp	2	1.17%	414	64	
				S	E
NR and Swiss Prot Database Neuronal Pas Domain Protein				24	38

Table 7: blastp of the ~390bp clones

Clone size	% of clones	% of total	Number of base pairs	# of amino acids from point of fusion	
390	6	3.53%	369	53	
				S	E
NR Database					
Solute carrier family 9 ((40-50% homology)				48	2e-06
Protein Tyrosine phosphatase				24	16
Myosin light chain kinase				24	16
Cell surface glycoprotein (CD-44)				23-22	48-62
Swiss Prot					
Testis specific basic protein				24	4.4
Protein tyrosine phosphatase				24	6.1
Myosin Light chain Kinase				24	6.6
CD-44 antigen Precursor				22	22
Serum response factor (SRF)					
Low density Lipoprotein receptor-related protein 1 precursor (LRP) apolipoprotein E receptor				21	43
Laminin α-2 chain precursor				21	51

Table 8: tblastx of EST database results:

Clone Size (bp)	% of total	# of base pairs	Candidate protein	S	E
1000	10.59	937	CDNA clone similar to Procollagen alpha2 (I) chain	278	2e-73
900	0.5	873	NCI_CGAP_Lu34 cDNA clone	104	7e-21
800	2.94	729	Artifact	-	-
620	17.6	575	NCI_CGAP_Lu28 cDNA clone similar tp Procollagen alpha2(I) chain	244	2e-63
607	17.05	576	Artifact	-	-
500	1.76	504	Genomic DNA, Chromosome 8q23 clone	182	4e-44
410	1.17	414	NCI_CGAP_Brn2 5 cDNA clone similar to sex-regulated protein Janus-A	156	4e-37
390	3.54	369	IL-5	126	3e-28
375	3.5	354	Artifact	-	-
350	15	327	Dipeptidyl carboxypeptidase1 (angiotensin I-converting enzyme)	27.2	2

blastn and tblastx results: Blast of human EST database with the translated nucleotide sequence downstream of phage coat protein in six reading frames took care of any early termination codons that may have been introduced due to frame shifts caused by sequencing errors since the entire nucleotide sequence beyond the point of fusion was used for homology search. This search showed that the most abundant clones (clones of 1000 and 600 bps) showed binding to sequences with a high score S to the α2 (I) chain of collagen I (Table 8). Interestingly, we reported that the α2(IV) chain of collagen IV bound with high affinity to pro-MMP-9 (11). Also a high score was obtained for clones showing homologies to binding with IL-5. Chance occurrence of these clones was remote as revealed by low E

values of 2e-73 and 3e-28. Clones of 350 bp size, representing 15% of the selected clones, showed homologies with dipeptidyl peptidase with a relatively modest Score of 27.2. Clones 800, 607 and 375 were artifacts since they showed homologies with the subjects representing DNA strands in reverse orientation albeit with 100% homology.

Discussion. The foregoing results have been obtained from screening of a library by specific protein-protein interactions. The search should have yielded proteins or peptides showing 100% homology and high scores. Looking at tables 2, 3,4, 5, 6 and 7, we find that the score values range anywhere between 20-36 on an average (S=48 for 390 and is an exception). These scores are low when compared to the scores obtained at the nucleotide level. One may attribute this fundamental dichotomy in the results obtained by protein and nucleotide blasts respectively to frame shifts caused by sequencing errors. Nevertheless, these differences make it difficult to draw any meaningful conclusions. In view of the constraints inherent in the technique alternative strategies may have to be employed for identifying MMP-9 binding proteins. We have initiated studies with the yeast two-hybrid system to search for MMP-9 binding proteins

KEY RESEARCH ACCOMPLISHMENTS

- Characterized the processing of MT1-MMP and described the major enzyme forms
- Reported the synergistic and enhancing effects of TIMP-2 and synthetic MMPIs in pro-MMP-2 activation
- Showed that TIMP-4 cannot promote pro-MMP-2 activation by MT1-MMP
- Demonstrated the localization of MMP-2 and MT1-MMP in caveolae
- Carried out a search for MMP-9 binding proteins using a phage display system

REPORTABLE OUTCOMES

Published papers.

1. Toth, M., Gervasi, D.C., Bernardo, M.M., Soloway, P.D., Wang, Z., Bigg, H.F., Overall, C.M., DeClerck, Y.A., Tschesche, H., Cher, M., Brown, S., Mobashery, S., and Fridman, R. (2000).

TIMP-2 acts synergistically with synthetic MMP inhibitors but not with TIMP-4 to enhance the MT1-MMP-dependent activation of pro-MMP-2. J. Biol. Chem., 275, 41415-41423.

2. Puyraimond, A, Fridman, R., Lemesle, M., Arbeille, B., and Menashi, S. (2001). MMP-2 colocalizes with caveolae on endothelial cell surface. Exp. Cell Res., 262, 28-36.

3. Hernandez-Barrantes, S., Shimura, Y., Soloway, P.D., Sang, Q-X.A., and Fridman, R. (2001). Differential roles of TIMP-4 and TIMP-2 in pro-MMP-2 activation by MT1-MMP. Bioch. Biophys. Res. Comm., 281, 126-130.

Abstracts.

1. Hernandez-Barrantes, S., Shimura, Y., Soloway, P.D., and Fridman, R. "Co-expression of TIMP-4 with MT1-MMP cannot promote pro-MMP-2 activation. Differential roles of TIMPs in regulation of pericellular proteolysis." Meeting of the International Metastasis Society, London, UK, September 2000.

The matrix metalloproteinases (MMPs) have been associated with tumor invasion and metastasis. Recent evidence suggests a role for the membrane type 1 (MT1)-MMP in metastasis and angiogenesis. MT1-MMP is a membrane bound MMP that is known to be the physiological activator of pro-MMP-2. A model for the activation of pro-MMP-2 has been proposed in which the catalytic domain of MT1-MMP binds to the N-terminal domain of TIMP-2 while TIMP-2 C-terminal domain binds to the hemopexin-like domain (HLD) of pro-MMP-2. Moreover, studies have shown that the amount of active MT1-MMP (57 kDa) on the surface is regulated by TIMP-2. Thus, TIMP-2 can act as a positive regulator of MMP activity. Like TIMP-2, TIMP-4 has been shown to bind to the HLD of pro-MMP-2. Thus, TIMP-4 can potentially participate in the activation of pro-MMP-2 by MT1-MMP. Here we co-expressed MT1-MMP with or without TIMP-2 or TIMP-4 in TIMP-2 null cells and examine their ability to promote pro-MMP-2 activation and accumulation of active MT1-MMP. Whereas TIMP-2 enhanced pro-MMP-2 activation by MT1-MMP, TIMP-4 had no effect. Interestingly, both inhibitors induced the accumulation of the 57-kDa active form of MT1-MMP in the cells consistent with their inhibitory activity. Co-expression of TIMP-4 with TIMP-2 inhibited pro-MMP-2 activation. These studies demonstrate that TIMP-4 is a true MT1-MMP inhibitor but is unable to participate in pro-MMP-2 activation. We propose that a balance between TIMP-2 and TIMP-4 in tumor tissues may be a critical factor in determining the invasive/degradative potential of tumor cells.

2. Toth, M. and Fridman, R. "MT-MMP-TIMP interactions regulate enzyme processing and activity on the cell surface." Gordon Research Conference on Matrix Metalloproteinases, Il Ciocco, Italy, May 2001.

Cell behavior is strictly dependent on the cell's ability to control its immediate microenvironment through surface proteolysis. The members of the MMP family play a pivotal role in pericellular proteolysis. We have been studying the surface regulation of two distinct MMPs, MT1-MMP and MMP-9, known for their role in cancer metastasis and angiogenesis. MT1-MMP activity on the surface is partly controlled by a delicate balance between trafficking and insertion of active enzyme to the plasma membrane and its turnover by autocatalytic processing. The concentration of active MT1-MMP on the cell surface is positively influenced by natural and synthetic MMP inhibitors (MMPIs), which sustain enzyme presence in the membrane and, under restricted conditions, may promote MT1-MMP-dependent activity. Under low inhibitor concentrations, active MT1-MMP is processed to an inactive membrane-tethered form of 44 kDa while the entire catalytic domain (MT1-MMP_{cat}) is shed into the extracellular space. Shedding of MT1-MMP_{cat} is significantly reduced by both TIMP-2 and TIMP-4 as well as by broad spectrum MMPIs consistent with being an autocatalytic event. Binding of TIMP-2 but not of TIMP-4 to MT1-MMP is also essential for pro-MMP-2 activation. However, MMPIs can enhance, synergistically, the effect of TIMP-2 on pro-MMP-2 activation. In contrast, TIMP-4 inhibits this effect. Structurally, neither the cytosolic tail nor the hinge region of MT1-MMP are necessary for pro-MMP-2 activation in the presence of TIMP-2 suggesting that these domains are not required for ternary complex formation and catalytic activity on the cell surface. Taken together these studies suggest that a delicate balance of MT-MMP, TIMPs and MMPIs can alter pericellular proteolysis and activation cascades by regulating the nature of membrane-tethered forms and the shedding of the catalytic domain.

3. Fridman, R. "Surface association and regulation of MT1-MMP and MMP-9: Tales of two enzymes and one dilemma." International Conference on Surface Proteases and Cancer, Palermo, Italy, May 2001.

Cell behavior is strictly dependent on the cell's ability to control its immediate microenvironment through surface proteolysis. The members of the MMP family play a pivotal role in pericellular proteolysis, which is partly determined by innate domain features in enzyme structure or by interactions with specific cell surface components. We have been studying the surface regulation of two distinct MMPs, MT1-MMP and MMP-9, known for their role in cancer metastasis and

angiogenesis. MT1-MMP activity on the surface is partly controlled by a delicate balance between trafficking and insertion of active enzyme to the plasma membrane and its turnover by autocatalytic processing. The concentration of active MT1-MMP on the cell surface is positively influenced by natural and synthetic MMP inhibitors, which sustain enzyme presence in the membrane and, under restricted conditions, may promote MT1-MMP-dependent activity. Under low inhibitor concentrations, MT1-MMP is processed to an inactive membrane-tethered form while the entire catalytic domain is shed into the extracellular space. These results suggest that MT1-MMP activity in the pericellular compartment is positively influenced by TIMPs and may involve both membrane-bound and soluble enzymes possibly with different biological functions. Accumulating evidence suggests that gelatinases (MMP-2 and MMP-9), which are secreted MMPs, can also associate with the cell surface. In the case of pro-MMP-2, surface association is mediated by an MT1-MMP/TIMP-2 complex, which promotes pro-MMP-2 activation by facilitating zymogen binding to the cell surface. Pro-MMP-9 has been identified on the surface of a variety of cells where it binds with high affinity. Several proteins have been described as potential "MMP-9 receptors." However, their significance for MMP-9 activity and inhibition is unclear. Furthermore, in certain cases, strict criteria for ligand-receptor association have not been clearly established. The role of surface binding in pro-MMP-9 activation is unknown since a surface activation mechanism of pro-MMP-9 has yet to be identified. Such a mechanism should take into account that solution phase activation of pro-MMP-9 is kinetically very efficient. These and other issues regarding MMP-9 surface binding and the dilemmas that they present for our understanding of pericellular proteolysis will be discussed.

4. Bernardo, M.M., Brown, S., Li, Z-H., Kotra, L.P., Tanaka, Y., Fridman, R. and Mobashery, S. "Potent and Selective Mechanism-Based Inhibition of Gelatinases." Gordon Research Conference on Matrix Metalloproteinases, Il Ciocco, Italy, May 2001.

The metalloproteases, MMPs, are essential to normal tissue remodeling processes and have also been implicated in pathological conditions such as arthritis, cardiovascular diseases and cancer. The gelatinases, MMP-2 and MMP-9, have been shown to play a key role in angiogenesis and tumor metastasis. Synthetic MMP inhibitors with therapeutic value for these conditions are highly sought. All the currently available inhibitors take advantage of chelation of the active site zinc ion and lack specificity. For this purpose we have resorted to the design of mechanism-based inhibitors for the gelatinases. We have designed, synthesized and kinetically characterized the first mechanism-based inhibitor for MMP-2 and MMP-9. The compound, (4-phenoxyphenylsulfonyl)methylthiirane, comprises a biphenyl moiety which fits in the gelatinases active site as demonstrated by energy-

minimization modeling of the enzyme-inhibitor complexes. This binding mode brings the sulfur of the thiirane group into the coordination sphere of the zinc ion. Zinc coordination is expected to render the carbon of the thiirane moiety electrophilic, making it susceptible of being modified by a nucleophile, possibly the carboxy group of the active site glutamic acid residue (Glu-404), leading to the formation of an ester bond and to irreversible enzyme inactivation. Kinetic characterization of enzyme inhibition by this compound using synthetic fluorogenic substrates, was consistent with the proposed mechanism for gelatinase inactivation. Slow binding inhibition was observed for both MMP-2 and MMP-9 with K_i values of 13.9 $\pm$ 0.4 and 600 $\pm$ 200 nM, respectively. In contrast the K_i values for the other MMPs tested , MMP-3, MMP-7 and MMP–1, are in the micromolar range. Analogous compounds with longer backbones for the point of attachment of the thiirane moiety, or the corresponding oxirane derivatives, served as poor competitive inhibitors at best, and there were cases of no inhibition. The collective results indicate high selectivity of the aforementioned compound for the inhibition of gelatinases and should serve as a paradigm for the design of inhibitors for other closely related enzymes.

CONCLUSIONS

1. Active MT1-MMP undergoes a process of autocatalysis resulting in the generation of a membrane-tethered species of 44 kDa without the catalytic domain. TIMP-2, TIMP-4 and synthetic MMPIs inhibit this autocatalytic processing inducing accumulation of the active species on the cell surface. Due partly to this process, there is enhanced activation of pro-MMP-2 and thus TIMP-2 and MMPIs can act synergistically to promote pro-MMP-2 activation by MT1-MMP. Thus, under certain conditions synthetic MMPIs can promote MT1-MMP-dependent pericellular proteolysis. These findings are relevant for understanding the potential effects of MMPIs in cancer tissues.

2. TIMP-4 cannot promote pro-MMP-2 activation by MT1-MMP in spite of its ability to bind pro-MMP-2. TIMP-4 also competes with TIMP-2 and thus inhibits its ability to promote pro-MMP-2 activation by MT1-MMP. This suggests the possibility that in breast tumor tissues, a balance between TIMP-2 and TIMP-4 may regulate the degree of pro-MMP-2 activation.

3. We have carried out phage display studies to search for pro-MMP-9 binding proteins. These studies have provided conflicting data as explained above. However, several interesting proteins have

been identified. The reliability of this method is unclear and therefore we are not confident of the findings.

REFERENCES

1. W. G. Stetler-Stevenson, L. A. Liotta, P. D. Brown, *Cancer Treat Res* **61**, 21-41 (1992).
2. K. J. Heppner, L. M. Matrisian, R. A. Jensen, W. H. Rodgers, *Am J Pathol* **149**, 273-82 (1996).
3. I. Stamenkovic, *Semin Cancer Biol* **10**, 415-33. (2000).
4. P. A. Andreasen, L. Kjoller, L. Christensen, M. J. Duffy, *Int J Cancer* **72**, 1-22 (1997).
5. W. G. Stetler-Stevenson, A. E. Yu, *Semin Cancer Biol* **11**, 143-53. (2001).
6. H. Sato *et al.*, *Nature* **370**, 61-5 (1994).
7. M. Polette *et al.*, *Virchows Arch* **428**, 29-35 (1996).
8. S. Hernandez-Barrantes *et al.*, *J Biol Chem* **275**, 12080-9. (2000).
9. R. Fridman *et al.*, *Biochem J* **289**, 411-6 (1993); T. R. Fuerst, P. L. Earl, B. Moss, *Mol Cell Biol* **7**, 2538-44 (1987).
10. M. W. Olson *et al.*, *J Biol Chem* **275**, 2661-8 (2000).
11. M. W. Olson *et al.*, *J Biol Chem* **273**, 10672-81 (1998).

APPENDICES

1. Toth, M., Gervasi, D.C., Bernardo, M.M., Soloway, P.D., Wang, Z., Bigg, H.F., Overall, C.M., DeClerck, Y.A., Tschesche, H., Cher, M., Brown, S., Mobashery, S., and Fridman, R. (2000). TIMP-2 acts synergistically with synthetic MMP inhibitors but not with TIMP-4 to enhance the MT1-MMP-dependent activation of pro-MMP-2. J. Biol. Chem., 275, 41415-41423.

2. Hernandez-Barrantes, S., Shimura, Y., Soloway, P.D., Sang, Q-X.A., and Fridman, R. (2001). Differential roles of TIMP-4 and TIMP-2 in pro-MMP-2 activation by MT1-MMP. Bioch. Biophys. Res. Comm., 281, 126-130.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 52, Issue of December 29, pp. 41415–41423, 2000
Printed in U.S.A.

Tissue Inhibitor of Metalloproteinase (TIMP)-2 Acts Synergistically with Synthetic Matrix Metalloproteinase (MMP) Inhibitors but Not with TIMP-4 to Enhance the (Membrane Type 1)-MMP-dependent Activation of Pro-MMP-2*

Received for publication, July 31, 2000, and in revised form, September 14, 2000
Published, JBC Papers in Press, September 20, 2000, DOI 10.1074/jbc.M006871200

Marta Toth‡, M. Margarida Bernardo‡, David C. Gervasi‡, Paul D. Soloway§, Zhiping Wang§, Heather F. Bigg¶, Christopher M. Overall¶, Yves A. DeClerck‖, Harald Tschesche, Michael L. Cher‡ ‡‡, Stephen Brown§, Shahriar Mobashery§§, and Rafael Fridman‡¶¶**

*From the ‡Departments of Pathology, ‡‡Urology and §§Chemistry, Wayne State University, Detroit, Michigan 48201, the §Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York 14263, the ¶Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada, the ‖Division of Hematology-Oncology and Department of Pediatrics, Children's Hospital Los Angeles, University of Southern California, Los Angeles, California 90027, and the **Department of Biochemistry, Faculty of Chemistry, University of Bielefeld, W-4800 Bielefeld 1, Germany*

The membrane-type 1 matrix metalloproteinase (MT1-MMP) has been shown to be a key enzyme in tumor angiogenesis and metastasis. MT1-MMP hydrolyzes a variety of extracellular matrix components and is a physiological activator of pro-MMP-2, another MMP involved in malignancy. Pro-MMP-2 activation by MT1-MMP involves the formation of an MT1-MMP·tissue inhibitors of metalloproteinases 2 (TIMP-2)·pro-MMP-2 complex on the cell surface that promotes the hydrolysis of pro-MMP-2 by a neighboring TIMP-2-free MT1-MMP. The MT1-MMP·TIMP-2 complex also serves to reduce the intermolecular autocatalytic turnover of MT1-MMP, resulting in accumulation of active MT1-MMP (57 kDa) on the cell surface. Evidence shown here in *Timp2*-null cells demonstrates that pro-MMP-2 activation by MT1-MMP requires TIMP-2. In contrast, a C-terminally deleted TIMP-2 (Δ-TIMP-2), unable to form ternary complex, had no effect. However, Δ-TIMP-2 and certain synthetic MMP inhibitors, which inhibit MT1-MMP autocatalysis, can act synergistically with TIMP-2 in the promotion of pro-MMP-2 activation by MT1-MMP. In contrast, TIMP-4, an efficient MT1-MMP inhibitor, had no synergistic effect. These studies suggest that under certain conditions the pericellular activity of MT1-MMP in the presence of TIMP-2 can be modulated by synthetic and natural (TIMP-4) MMP inhibitors.

Proteolytic degradation of extracellular matrix (ECM)[1] is a fundamental aspect of cancer development and a key event in tumor-induced angiogenesis and tumor metastasis. A major group of enzymes responsible for ECM degradation in cancer tissue is the matrix metalloproteinase (MMP) family (1–4). The MMPs are zinc-dependent multidomain endopeptidases that, with few exceptions, share a basic structural organization comprising propeptide, catalytic, hinge, and C-terminal (hemopexin-like) domains (1, 5). All MMPs are produced in a latent form (pro-MMP) requiring activation for catalytic activity, a process that is usually accomplished by proteolytic removal of the propeptide domain. Once activated, all MMPs are specifically inhibited by a group of endogenous tissue inhibitors of metalloproteinases (TIMPs) that bind to the active site, inhibiting catalysis (1). Over the last 5 years, the MMP family has been expanded to include a new subfamily of membrane-tethered MMPs known as membrane-type MMPs (MT-MMPs), which to date includes six members (6–12). The MT-MMPs, with the exception of MT4-MMP, are unique because they are anchored to the plasma membrane by means of a hydrophobic stretch of approximately 20 amino acids, leaving the catalytic domain exposed to the extracellular space. This organization makes the MT-MMPs perfectly suited for regulation of pericellular proteolysis. MT1-MMP (MMP-14) was the first member of the MT-MMP family to be discovered and has been shown to be the major physiological activator of pro-MMP-2 (gelatinase A) on the cell surface (6, 12). The role of MT1-MMP in pericellular proteolysis is not restricted to pro-MMP-2 activation, since MT1-MMP is a multifunctional enzyme that can also degrade a variety of ECM components (13–16) and hence can play a direct role in ECM turnover. MT1-MMP has been recently shown to be the first member of the MMP family indispensable for normal growth and development, since mice deficient in MT1-MMP exhibit a variety of connective tissue pathologies and a short life span (17, 18). Furthermore, both MMP-2 (19) and MT1-MMP (20–26) have been associated with metastatic potential in many human cancers, angiogenesis (27), and enhanced tumor cell invasion in experimental systems (28–31). This has raised considerable interest in understanding the regulation of these MMPs because they represent an important target for development of novel drugs aimed at inhibiting tumor metastasis and angiogenesis (3, 32, 33).

Studies on the mechanism of activation of pro-MMP-2 by MT1-MMP revealed a complex role for TIMP-2 in this process. A model for the activation of pro-MMP-2 has been proposed in which the catalytic domain of MT1-MMP binds to the N-termi-

* This work was supported by National Institutes of Health Grant CA-61986-06 and Department of Defense Grant DAMD17-99-1-9440 (to R. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶¶ To whom all correspondence should be addressed: Dept. of Pathology, Wayne State University, 540 E. Canfield Ave. Detroit, MI 48201. Tel.: 313-577-1218; Fax: 313-577-8180; E-mail: rfridman@med.wayne.edu.

[1] The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; MT-MMP, membrane type MMP; MMPI, MMP inhibitor; TIMP, tissue inhibitor of metalloproteinase; PAGE, polyacrylamide gel electrophoresis; pAb, polyclonal antibody; pfu, plaque-forming units; DMEM, Dulbecco's modified Eagle's medium.

nal portion of TIMP-2, leaving the negatively charged C-terminal region of TIMP-2 available for the binding of the hemopexin-like domain of pro-MMP-2 (12, 34–38). This ternary complex has been suggested to cluster pro-MMP-2 at the cell surface near a residual TIMP-free active MT1-MMP molecule, which is thought to initiate activation of the bound pro-MMP-2. Pro-MMP-2 activation would occur only at low TIMP-2 concentrations relative to MT1-MMP, which would permit availability of active MT1-MMP to activate the pro-MMP-2 bound in the ternary complex (39). Thus, under restricted conditions, TIMP-2 is thought to promote the activation process by acting as a molecular link between MT1-MMP and pro-MMP-2. We have recently shown that TIMP-2, besides its role in ternary complex formation, has direct and critical effects on MT1-MMP processing, which influence the profile and spatial localization of MT1-MMP forms (40). Biochemical and cellular evidence showed that binding of TIMP-2 to active MT1-MMP (57 kDa) inhibits autocatalytic degradation, leading to accumulation of active MT1-MMP on the cell surface. In the absence of TIMP-2, MT1-MMP is rapidly processed to a 44-kDa membrane-bound inactive enzyme (40, 41). Thus, under controlled conditions, TIMP-2 may act as a positive regulator of MT1-MMP activity by promoting the availability of active MT1-MMP on the cell surface and consequently may support pericellular proteolysis. Since some of the effects of TIMP-2 on MT1-MMP activities are related to its inhibitory activity, we wished to examine the effects of synthetic and physiological MMP inhibitors (MMPIs) on MT1-MMP processing and its ability to promote pro-MMP-2 activation with TIMP-2. Although several types of MMPIs have been developed (3, 32, 33, 42–47), little is known about their effects on the processing and activity of membrane-tethered MMPs, which exhibit unique properties. Here we show for the first time that synthetic MMPIs and a C-terminally truncated TIMP-2 but not TIMP-4, which inhibit MT1-MMP activity, act synergistically with TIMP-2 to promote pro-MMP-2 activation by MT1-MMP. These studies demonstrate the complexity of MT1-MMP regulation and provide new insights into the roles of TIMP-2, TIMP-4, and MMPIs in this process.

EXPERIMENTAL PROCEDURES

Cell Culture—Nonmalignant monkey kidney epithelial BS-C-1 (CCL-26) and human fibrosarcoma HT-1080 (CCL-121) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. HeLa S3 cells were obtained from ATCC (CCL-2.2) and grown in suspension in MEM Spinner medium (Quality Biologicals, Inc., Gaithersburg, MD) supplemented with 5% horse serum. All other tissue culture reagents were purchased from Life Technologies, Inc.

Isolation of Immortalized Timp2 Mutant Mouse Fibroblasts—Adult skin fibroblast cells were isolated from heterozygous (+/−) *Timp2* mutant mice and immortalized by retroviral infection using a Ha-*ras* and v-*myc*-producing, replication-defective retrovirus as described previously (48). A G418 selection protocol was used to select for homozygous *Timp2* (−/−) mutant cells from the immortalized (+/−) mutant clone as described (49). Detailed methods for the isolation and selection of the immortalized (+/−) and (−/−) isogenic cells will be reported elsewhere. The homozygous and heterozygous *Timp2* mutant cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics.

Recombinant Vaccinia Viruses—The production of the recombinant vaccinia virus (vTF7–3) expressing bacteriophage T7 RNA polymerase has been described by Fuerst *et al.* (50). Recombinant vaccinia viruses expressing either human pro-MMP-2, TIMP-2, or MT1-MMP under control of the T7 promoter were obtained by homologous recombination as described previously (35, 40, 51).

Recombinant Proteins, Synthetic MMP Inhibitors, and Antibodies—Human recombinant pro-MMP-2, TIMP-2, and TIMP-1 were expressed in HeLa S3 cells infected with the appropriate recombinant vaccinia viruses and purified to homogeneity, as described previously (52). Human recombinant TIMP-4 was expressed in baby hamster kidney cells and purified from the conditioned medium by sequential application to Red Sepharose, phenyl-Sepharose, Q Sepharose, and Zn^{2+}-charged chelating Sepharose columns as described.[2] A C-terminally truncated human TIMP-2 ending at Cys^{128} (Δ-TIMP-2) was constructed and expressed in mammalian cells as described previously (53). The concentrations of TIMP-2 and Δ−TIMP-2 were determined by active-site titration with MMP-2. A $Cys^{1} \rightarrow Ala$ TIMP-2 mutant (Ala + TIMP-2) was kindly provided by Dr. W. G. Stetler-Stevenson (NCI, National Institutes of Health) (54). A recombinant catalytic domain of human MT1-MMP (MT1-MMP_{cat}) comprising residues Ile^{114} to Ile^{318} was expressed in *Escherichia coli*, purified, and characterized as described previously (55). The concentration of the MT1-MMP_{cat} enzyme was determined by active-site titration with recombinant TIMP-2 as described (56). Batimastat (BB-94) and marimastat (BB-2516), two hydroxamate-based MMP inhibitors (33, 45, 47), and BB-2116, a boronate-containing MMP inhibitor, were obtained from British Biotech (Annapolis, MD). The mechanism-based MMP inhibitor SB-3CT was synthesized and characterized as previously reported (42). Stock solutions of marimastat (1 mM), batimastat (1 mM), BB-2116 (20 mM), and SB-3CT (30 mM) were prepared in Me_2SO. The rabbit anti-TIMP-2 polyclonal antibody (pAb) and the anti-TIMP-2 monoclonal antibody CA-101 were previously described (57). The rabbit pAb 437 to MT1-MMP (40, 58) has been previously described. The rabbit pAb 160 to MT1-MMP (40, 59) and the rabbit pAb to human TIMP-4 were a generous gift from Dr. Amy Sang (Florida State University, Tallahassee, FL).

Expression of MT1-MMP by Vaccinia Infection—To express MT1-MMP, confluent cultures of BS-C-1 or *Timp2* mutant cells in 6- or 12-well plates were co-infected with 5–10 pfu/cell each of vTF7–3 and vT7-MT1 viruses for 45 min in infection medium (DMEM plus 2.5% fetal bovine serum and antibiotics) at 37 °C. As control, the cells were infected only with the vTF7–3 virus as described (40).

Natural and Synthetic Inhibitor Treatment and Pro-MMP-2 Activation—After infection, the media were aspirated, and the cells were rinsed with serum-free DMEM and replaced with fresh serum-free DMEM supplemented with or without various doses of purified human recombinant TIMP-2. After various times at 37 °C, the media were aspirated; the cells were rinsed with DMEM and then incubated (15–30 min, 37 °C) in fresh media supplemented with 10 nM pro-MMP-2. The media were then collected, and the cells were rinsed twice with cold phosphate-buffered saline and solubilized in cold lysis buffer (25 mM Tris-HCl (pH 7.5), 1% IGEPAL CA-630, 100 mM NaCl, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride). The lysate fractions were analyzed for pro-MMP-2 activation by gelatin zymography and/or immunoblot analysis for assessment of MT1-MMP forms. To examine the effects of synthetic MMP inhibitors, Δ−TIMP-2, Ala + TIMP-2, and TIMP-4 on TIMP-2-dependent activation of pro-MMP-2, the MT1-MMP-infected cells were treated (16 h, 37 °C) with the appropriate MMP inhibitors (various doses) diluted in serum-free DMEM. Then the media were aspirated, and the cells were rinsed with DMEM. TIMP-2 (10 nM) was then added to the cells in serum-free DMEM for a 5–30-min incubation at 37 °C. The media were aspirated followed by a wash with DMEM to remove unbound TIMP-2. The cells were then incubated (15 min, 37 °C) with serum-free DMEM supplemented with 10 nM pro-MMP-2. Analysis of pro-MMP-2 activation and of the profile of MT1-MMP forms in the cell lysates were carried out as described below.

Gelatin Zymography and Immunoblot Analysis—Gelatin zymography was performed using 10% Tris-glycine SDS-polyacrylamide gels containing 0.1% gelatin. Briefly, samples of lysates or media were mixed with Laemmli sample buffer (60) without reducing agents and without heating and then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (61). For immunoblot analysis, the cell lysates were subjected to reducing SDS-PAGE following by transfer to a nitrocellulose membrane essentially as described (61). Detection of the immune complexes was performed using the enhanced chemiluminescence system (Pierce) according to the manufacturer's instructions.

Enzyme Inhibition Studies—MT1-MMP_{cat} activity was monitored with the fluorescence-quenched substrate $MOCAcPLGLA_2pr(Dnp)$-$AR-NH_2$ (62). Fluorescence was measured with a Photon Technology International (PTI) spectrofluorometer interfaced to a Pentium computer, equipped with the RatioMaster™ and FeliX™ hardware and software, respectively. The cuvette compartment was maintained at 25 °C. Excitation and emission passes of 1 and 3 nm, respectively, were used.

[2] Bigg, H. F., Morrison, C. J., Butler, G. S., Bogoyevitch, M.A., Wang, Z., Soloway, P. D., and Overall, C. M., submitted for publication.

Substrate hydrolysis was monitored at emission and excitation wavelengths of 328 and 393 nm, respectively. Fluorescence measurements were taken every 4 s. Less than 10% hydrolysis of the fluorogenic substrate was monitored, as described by Knight (62). For slow binding inhibition, progress curves were obtained by adding enzyme (0.5 nM) to a mixture of fluorogenic substrate (7 μM) and varying concentrations of inhibitor in buffer R (50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$, 0.01% Brij-35, and 1–5% Me_2SO; final volume 2 ml) in acrylic cuvettes with stirring and monitoring the increase in fluorescence with time for 15–30 min. The progress curves were nonlinear least squares fitted to Equation 1 (63),

$$F = v_s t + I(v_o - v_s)\,(1 - \exp(-kt))/k + F_0 \qquad \text{(Eq. 1)}$$

where v_0 represents the initial rate, v_s is the steady state rate, k is the apparent first order rate constant characterizing the formation of the steady-state enzyme-inhibitor complex, and F_0 is the initial fluorescence, using the program SCIENTIST (MicroMath Scientific Software, Salt Lake City, UT). The obtained k values, v_0, and v_s were further analyzed according to Equations 2 and 3 for a one-step association mechanism.

$$k = k_{off} + k_{on}\,[I]/(1 + [S]/K_m) \qquad \text{(Eq. 2)}$$

$$(v_o - v_s)/v_s = [I]/(K_i(1 + [S]/K_m)) \qquad \text{(Eq. 3)}$$

The K_m and k_{cat} values for the reaction of MT1-MMP$_{cat}$ with the fluorogenic substrate were determined to be 6.9 ± 0.6 μM and 0.67 ± 0.03 s^{-1}, respectively. Intercept and slope values, obtained by linear regression of the k *versus* inhibitor concentration plot (Equation 2), yielded the association and dissociation rate constants k_{on} and k_{off}, respectively, and the inhibition constant K_i (k_{off}/k_{on}). Alternatively, K_i was determined from the slope of the $(v_0 - v_s)/v_s$ *versus* [I] plot according to Equation 3. The dissociation rate constant was determined independently from the enzyme activity recovered after dilution of a preformed enzyme-inhibitor complex. To this end, typically 50 nM of enzyme was incubated with 80 nM of inhibitor for a sufficient time to reach equilibrium (>45 min) at 25.0 °C. The complex was diluted 400-fold into 2 ml of buffer R containing fluorogenic substrate (10 μM final concentration). Recovery of enzyme activity was monitored for ~60 min. The fluorescence *versus* time trace was fitted, using the program SCIENTIST, to Equation 4,

$$F = v_s t + (v_o - v_s)\,(1 - \exp(-k_{off} t))/k_{off} + F_0 \qquad \text{(Eq. 4)}$$

where v_0 represents the initial rate (very small), v_s is the rate observed when the *EI* complex is completely dissociated, and k_{off} is the first order rate constant of *EI* dissociation. In light of the slow dissociation of the MT1-MMP$_{cat}$-TIMP-2 complex, the direct analysis of the k_{off} parameter for the wild type TIMP-2 was not possible and was estimated based on a 10-fold difference observed between the slopes of the linear portions of the dissociation curves for the complexes of MT1-MMP$_{cat}$ with Δ-TIMP-2 (steady state rate) and wild type TIMP-2. For competitive inhibition, initial rates were obtained by adding enzyme (0.5 nM) to a mixture of fluorogenic substrate (7 μM) and varying concentrations of inhibitor in buffer R (final volume 1 ml) in quartz semimicro cuvettes and monitoring the increase in fluorescence with time for 5–10 min. The initial velocities were determined by linear regression analysis of the fluorescence *versus* time traces using FeliX™. The initial rates were fitted to Equation 5 (64),

$$v_i/v_0 = (K_m + [S])/(K_m(1 + [I]/K_i) + [S]) \qquad \text{(Eq. 5)}$$

where v_i and v_0 represent the initial velocity in the presence and absence of inhibitor, respectively, using the program SCIENTIST.

RESULTS

Pro-MMP-2 Activation and MT1-MMP Processing in Timp2 Mutant Cells—Using a vaccinia expression system, we have recently shown that immortalized monkey kidney epithelial BS-C-1 cells infected to express MT1-MMP could activate pro-MMP-2 (40). Under these conditions, this process appeared to be independent of TIMP-2, since in infected BS-C-1 cells expression of the endogenous inhibitor was significantly suppressed (35, 40, 51). However, these studies were inconclusive in regard to the requirement of TIMP-2 for pro-MMP-2 activation, since a residual amount of endogenous inhibitor could not be ruled out. To establish the importance of TIMP-2 in the activation of pro-MMP-2 by MT1-MMP, we used homozygous (−/−) and heterozygous (+/−) *Timp2* mutant mouse fibroblasts (65) that were immortalized by retroviral infection. We tested the expression of TIMP-2 in both cell types by immunoblot analysis. As shown in Fig. 1*A*, TIMP-2 was only detected in the *Timp2* (+/−) mutant cells, as reported with the primary fibroblast cells (65). The cells were then tested for activation of exogenous pro-MMP-2 after treatment with concanavalin A (66), and neither cell variant activated pro-MMP-2 regardless of the presence of TIMP-2 (data not shown), suggesting a low level of endogenous MT1-MMP expression. We therefore infected the *Timp2* (+/−) and (−/−) mutant cells to express MT1-MMP using the recombinant vaccinia virus (vT7-MT1) and the T7 RNA polymerase virus (vTF7–3) (40). As control, the cells were infected with the vTF7–3 virus alone. MT1-MMP expression (60- and 44-kDa bands) was detected only in the cells infected to express MT1-MMP (Fig. 1*B*, *lane 2*) but not in the control-infected cells (Fig. 1*B*, *lane 1*) in both cell types (only the homozygous cells are shown in Fig. 1*B*).

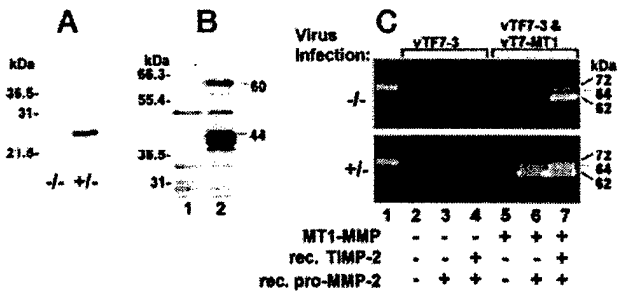

FIG. 1. **TIMP-2 expression and pro-MMP-2 activation in *Timp2* (+/−) and (−/−) mutant cells.** *A*, concentrated serum free-conditioned medium of −/− and +/− *Timp2* mutant cells was subjected to reducing 15% SDS-PAGE followed by immunoblot analysis using a specific pAb to TIMP-2. Detection of the antigen was performed by enhanced chemiluminescence. *B*, (−/−) *Timp2* mutant cells were infected with 10 pfu/cell of vTF7–3 (*lane 1*) or co-infected with 10 pfu/cell each of vTF7–3 and vT7-MT1 (*lane 2*) vaccinia viruses. Twenty-four hours postinfection, the cells were solubilized in lysis buffer and subjected to reducing 10% SDS-PAGE followed by immunoblot analysis using the pAb 437 to MT1-MMP. *C*, *Timp2* (−/−) and (+/−) mutant cells in six-well plates were infected with 10 pfu/cell of vTF7–3 (*lanes 2–4*) or coinfected with 10 pfu/cell each of vTF7–3 and vT7-MT1 (*lanes 5–7*) recombinant vaccinia viruses. After infection, the cells were incubated (16 h, 37 °C) with (+) or without (−) 10 nM recombinant TIMP-2 in serum-free DMEM (1 ml/well) followed by a gentle wash to remove unbound inhibitor. Then some wells received (*rec.*) 10 nM pro-MMP-2 (*lanes 3*, *4*, *6*, and *7*) in DMEM (1 ml/well) followed by a 30-min incubation at 37 °C. The cells were lysed in lysis buffer, and pro-MMP-2 activation was monitored by gelatin zymography. *Lane 1* in *C* shows the pro-MMP-2 added to the media. *72 kDa*, pro-MMP-2; *64 kDa*, intermediate form; *62 kDa*, active MMP-2.

The ability of the expressed MT1-MMP to promote pro-MMP-2 activation with or without TIMP-2 in this cellular system was examined by gelatin zymography of the lysate fraction. To this end, after infection, the cells were incubated with or without exogenous TIMP-2, washed to remove unbound inhibitor, and incubated with exogenous pro-MMP-2. The latter was added, since both the (+/−) and (−/−) *Timp2* mutant cells do not produce detectable pro-MMP-2 (Fig. 1*C*, *lanes 2* and *5*). As shown in Fig. 1*C*, the (−/−) *Timp2* mutant cells expressing MT1-MMP activated pro-MMP-2 only after the addition of exogenous TIMP-2 (Fig. 1*C*, *lane 7*). In contrast, the *Timp2* (+/−) mutant cells expressing MT1-MMP activated pro-MMP-2 regardless of exogenous TIMP-2 presence (Fig. 1*C*, *lane 6*, without TIMP-2; *lane 7*, with TIMP-2). Control-infected homozygous and heterozygous *Timp2* mutant cells consistently failed to activate pro-MMP-2 regardless of exogenous TIMP-2

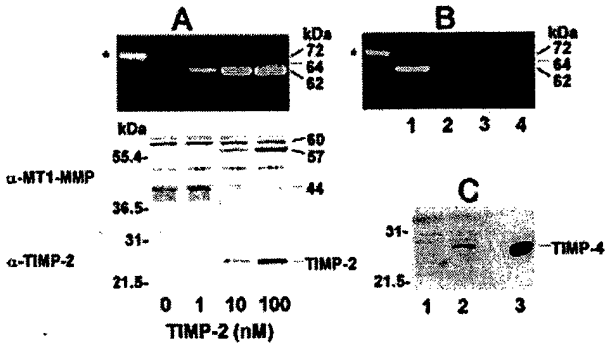

FIG. 2. **Effect of TIMP-2, Δ-TIMP-2, TIMP-4, and TIMP-1 on pro-MMP-2 activation in *Timp2* (−/−) mutant cells.** *A*, confluent *Timp2* (−/−) mutant cells were co-infected to express MT1-MMP as described in the legend to Fig. 1 and incubated (16 h, 37 °C) with TIMP-2 (0–100 nM). After a gentle wash, the cells were incubated with 10 nM pro-MMP-2 for 15 min at 37 °C. The lysates were subjected to gelatin zymography (nonreducing conditions) and reducing 10% and 15% SDS-PAGE for detection of MT1-MMP and TIMP-2, respectively, by immunoblot analysis with pAb 437 to MT1-MMP and monoclonal antibody CA-101 to TIMP-2 followed by detection with enhanced chemiluminescence. The ~50-kDa band is nonspecific. *B*, *Timp2* (−/−) mutant cells infected to express MT1-MMP were incubated (16 h, 37 °C) with 2.5 nM TIMP-2 (*lane 1*), 25 nM Δ-TIMP-2 (*lane 2*), 10 nM TIMP-4 (*lane 3*), or 25 nM TIMP-1 (*lane 4*). After the incubation, the media were aspirated, and the cells were washed with DMEM followed by the addition of serum-free DMEM containing 10 nM pro-MMP-2. After a 15-min incubation at 37 °C, pro-MMP-2 activation was determined in the cell lysate fraction by gelatin zymography. The *asterisks* show the pro-MMP-2 added to the media. *C*, *Timp2* (−/−) mutant cells infected to express MT1-MMP were incubated (16 h, 37 °C) without (*lane 1*) or with (*lane 2*) 10 nM exogenous recombinant TIMP-4. The cell lysates were subjected to reducing 10% SDS-PAGE followed by immunoblot analysis using a specific rabbit pAb to TIMP-4. *Lane 3* shows 10 ng of the purified recombinant TIMP-4.

presence (Fig. 1*C*, *lane 3*, without TIMP-2; *lane 4* with TIMP-2). Taken together, these studies establish the importance of TIMP-2 for the MT1-MMP-dependent activation of pro-MMP-2 and are in agreement with recent *in vitro* and *in vivo* studies (65, 67) with *Timp2* mutant fibroblasts and mice, respectively.

We have previously shown that TIMP-2 regulates the turnover of MT1-MMP on the cell surface by binding to the active form of the enzyme (40). This process induces the accumulation of active MT1-MMP (57 kDa) on the cell surface and concomitantly decreases the amount of a membrane-tethered 44-kDa form of MT1-MMP (40). N-terminal sequencing data demonstrated that the 57-kDa species starts at Tyr^{112} and the 44-kDa species starts at Gly^{285}; thus the latter represents an inactive enzyme form (40). To examine the relationship between pro-MMP-2 activation and MT1-MMP processing in the *Timp2*-null cell system, the homozygous *Timp2* mutant cells expressing MT1-MMP were analyzed for pro-MMP-2 activation and MT1-MMP forms as a function of TIMP-2 concentration. As shown in Fig. 2*A* (zymogram), as little as 1 nM TIMP-2 induced pro-MMP-2 activation as monitored in the cell lysate fraction. The lysates were also analyzed for MT1-MMP forms and TIMP-2 by immunoblot analyses (Fig. 2*A*, immunoblots). These studies show that overnight exposure to TIMP-2, at doses of >10 nM, induce a detectable accumulation of the 57-kDa species concomitantly with a reduction in the inactive 44-kDa form of MT1-MMP. Without TIMP-2 and at doses of 1 nM TIMP-2, the major species detected were the 60-kDa (pro-MT1-MMP) and the 44-kDa species. A minor 63-kDa protein represents the pro-MT1-MMP with the signal peptide,[3] and the ~50-kDa protein is a nonspecific band. TIMP-2 was also detected in the cell lysates (Fig. 2*A*, immunoblot α-TIMP-2) consistent with the association of the exogenous TIMP-2 with the MT1-MMP-expressing cells (38).

Importance of Ternary Complex on Pro-MMP-2 Activation by MT1-MMP in the Timp2-Null Cellular System—Previous studies demonstrated that the formation of an MT1-MMP·TIMP-2·pro-MMP-2 (ternary) complex on the cell surface is required for pro-MMP-2 activation (12, 36, 39). To demonstrate the importance of this complex in the *Timp2* null cellular system, we used a C-terminally truncated form of TIMP-2, Δ-TIMP-2, incapable of binding pro-MMP-2 (data not shown) and therefore unable to form the ternary complex (36, 53). In addition, we tested recombinant TIMP-4, known to bind to pro-MMP-2 (68). Due to their lower affinity for MT1-MMP, Δ-TIMP-2 (shown in Table I) and TIMP-1 (36, 69) were added to the homozygous cells at concentrations 10-fold higher than that of wild type TIMP-2. As shown in the zymogram of Fig. 2*B*, 2.5 nM TIMP-2 (Fig. 2*B*, *lane 1*) efficiently promoted pro-MMP-2 activation. In contrast, Δ-TIMP-2 (Fig. 2*B*, *lane 2*; 25 nM) and TIMP-1 (Fig. 2*B*, *lane 4*; 25 nM) failed to induce pro-MMP-2 activation. Interestingly, exogenous TIMP-4 also had no effect on activation (Fig. 2*B*, *lane 3*; 1–100 nM, only 10 nM shown), consistent with the results of Bigg *et al.*[2] In addition, co-expression of MT1-MMP with TIMP-4 in the (−/−) *Timp2* mutant cells using the vaccinia expression system had no effect on pro-MMP-2 activation.[4] Immunoblot analysis demonstrated the cell association of the exogenous TIMP-4 with (−/−) *Timp2* mutant cells expressing MT1-MMP (Fig. 2*C*, *lane 2*) suggesting the binding of TIMP-4 to MT1-MMP. Taken together, these results demonstrate that only full-length TIMP-2 can promote the MT1-MMP-dependent activation of pro-MMP-2 in a process that is dependent on ternary complex formation.

TIMP-2 and MMP Inhibitors Act Synergistically to Enhance Pro-MMP-2 Activation—Previous studies suggested that, in addition to ternary complex formation, the enhancing effect of TIMP-2 on pro-MMP-2 activation was the result of a specific inhibition of MT1-MMP autocatalytic turnover on the cell surface (40). Indeed, TIMP-2 induces the accumulation of the 57-kDa form of MT1-MMP (shown in Fig. 2*A*). It was hypothesized that at low inhibitor concentrations relative to MT1-MMP and continuous enzyme synthesis by the cells, this process would slow down enzyme turnover, generating a fraction of inhibitor-free active MT1-MMP and hence increase pericellular proteolysis (40). Since this effect is due to inhibition of MT1-MMP activity, we hypothesized that synthetic MMPIs may mimic TIMP-2 in its ability to reduce MT1-MMP turnover. We asked whether reduction of MT1-MMP autocatalytic turnover by MMPIs together with ternary complex formation by TIMP-2 would enhance pro-MMP-2 activation when compared with activation promoted by TIMP-2 alone.

To test this hypothesis, the *Timp2* (−/−) null cells were incubated overnight with a variety of synthetic and natural MMPIs to inhibit MT1-MMP autocatalysis and induce accumulation of the active 57-kDa species. The cells were then washed to remove excess unbound inhibitors and then exposed to TIMP-2 to generate the ternary complex. After a rinse to remove unbound TIMP-2, the cells received pro-MMP-2. We tested the effects of marimastat and batimastat, two hydroxamate-based inhibitors (33, 45, 47); SB-3CT, a mechanism-based inhibitor (42); and Δ-TIMP-2 (53). As shown in the zymogram of Fig. 3, administration of TIMP-2 alone for 5 min was sufficient to promote pro-MMP-2 activation, as expected (Fig. 3, *lane 2*). However, in the cells pretreated with a 1 μM concentration of either marimastat (Fig. 3, *lane 3*) or batimas-

[3] M. Toth, unpublished results.

[4] S. Hernandez-Barrantes, Y. Shimura, and R. Fridman, unpublished results.

TABLE I

Association, dissociation, and inhibition constants for MT1-MMP$_{cat}$ interactions with natural and synthetic MMP inhibitors

MT1-MMP$_{cat}$ (0.5 nM) was added to a solution of MOCAcPLGLA$_2$pr(Dnp)-AR-NH$_2$ (7 μM) and varying concentrations of inhibitor in buffer R at 25.0 °C. Substrate hydrolysis was monitored at excitation and emission wavelengths of 328 and 393 nm, respectively, for up to 30 min. To determine the dissociation rate constants, k_{off}, a mixture of enzyme (50 nM) and inhibitor (80 nM) in buffer R was incubated for ~1 h at 25 °C and diluted 400-fold in a solution of fluorogenic substrate (10 μM) in the same buffer containing 1% Me_2SO. Substrate hydrolysis was monitored for up to 1 h. The kinetic parameters were evaluated as described under "Experimental Procedures." Analogous results were obtained from at least two independent experiments.

Inhibitor	k_{on}	k_{off}	K_i
	$M^{-1}\ s^{-1}$	s^{-1}	nM
Wild type TIMP-2	$(2.74 \pm 0.14) \times 10^6$	2×10^{-4a}	0.07
Δ-TIMP-2	$(2.68 \pm 0.12) \times 10^6$	$(1.95 \pm 0.03) \times 10^{-3}$	0.73 ± 0.03
MARIMASTAT			2.1 ± 0.5
BATIMASTAT			3.4 ± 0.1
BB-2116			8 ± 1
SB-3CT			110 ± 11

[a] Estimated value based on a 10-fold difference between the slopes of the linear portions of the dissociation curves for the complexes of MT1-MMP$_{cat}$ with Δ-TIMP-2 (steady state rate) and wild type TIMP-2.

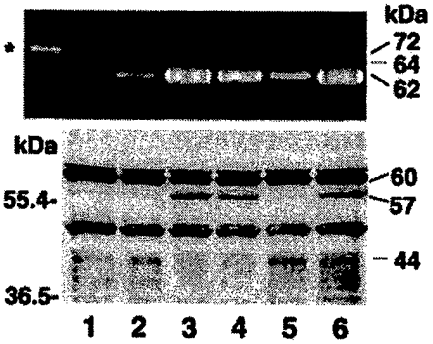

FIG. 3. **Synthetic MMPIs enhance pro-MMP-2 activation by MT1-MMP in the presence of TIMP-2.** The *Timp2* (−/−) mutant cells in 12-well plates were co-infected to express MT1-MMP as described in the legend to Fig. 1. After the infection, the media were aspirated and replaced with serum-free DMEM (1 ml/well) supplemented with 1 μM marimastat (*lane 3*), 1 μM batimastat (*lane 4*), 1 μM SB-3CT (*lane 5*), or 100 nM Δ-TIMP-2 (*lane 6*) followed by a 16-h incubation at 37 °C. Some wells received DMEM without inhibitors (*lanes 1* and *2*). The cells were washed once to remove excess inhibitors and then incubated with DMEM (1 ml/well) supplemented without (*lane 1*) or with (*lanes 2–6*) 10 nM TIMP-2 for 5 min at 37 °C. The media were then aspirated and replaced with fresh DMEM containing 10 nM pro-MMP-2. After 15 min at 37 °C, the cells were rinsed with phosphate-buffered saline and solubilized in lysis buffer. The lysates were analyzed for pro-MMP-2 activation and MT1-MMP forms by gelatin zymography and immunoblot analysis, respectively. The *asterisk* shows the pro-MMP-2 added to the media. The ~50-kDa band is nonspecific. This experiment was repeated at least three times with similar results.

tat (Fig. 3, *lane 4*) or 100 nM Δ-TIMP-2 (Fig. 3, *lane 6*), a significant increase in active MMP-2 was observed. SB-3CT at doses of 1 μM showed a modest effect (*lane 5*) when compared with the other synthetic MMPIs. Analysis of the lysate fractions for MT1-MMP forms by immunoblotting revealed accumulation of the 57-kDa species after treatment with marimastat, batimastat, and Δ-TIMP-2 (Fig. 3, *lanes 3*, *4*, and *6*, respectively) but not with SB-3CT (Fig. 3, *lane 5*). However, Δ-TIMP-2 was less efficient in inhibiting MT1-MMP conversion to the 44-kDa form (Fig. 3, *lane 6*, immunoblot). In the absence of TIMP-2, no activation was detected (Fig. 3, *lane 1*, zymogram).

Since the relationship between inhibition of MT1-MMP autocatalytic turnover and the existence of inhibitor-free MT1-MMP is dependent on the inhibitor concentration and affinity, we used inhibitor doses ranging from 0 to 500 nM to further examine the differential effects of marimastat and SB-3CT on pro-MMP-2 activation. We also tested BB-2116, a boronate-containing MMP inhibitor. In addition, we asked whether TIMP-4 would act synergistically with TIMP-2 in pro-MMP-2 activation by MT1-MMP. TIMP-4 inhibits MT1-MMP activity but cannot form a ternary complex with MT1-MMP and pro-MMP-2.[2] As shown in the zymogram of Fig. 4*A*, SB-3CT had no effect on pro-MMP-2 activation at doses of 4, 20, and 500 nM (Fig. 4*A*, *lanes 5* and *6*; only 20 and 500 nM, respectively, are shown). In contrast, as little as 20 nM marimastat (Fig. 4*A*, *lane 7*) had a noticeable effect on pro-MMP-2 activation when compared with TIMP-2 alone (Fig. 4*A*, *lane 2*). BB-2116 exhibited enhancing effects with TIMP-2 at doses of 500 nM (Fig. 4*A*, *lane 4*). Consistently, both marimastat (Fig. 4*A*, *lanes 7* and *8*) and BB-2116 (Fig. 4*A*, *lane 4*) induced accumulation of the 57-kDa form of MT1-MMP. Pretreatment of the cells with TIMP-4 (0–100 nM) followed by TIMP-2 addition, had no enhancing effect on pro-MMP-2 activation (Fig. 4*B*). Interestingly, TIMP-4 induced the accumulation of 57-kDa form of MT1-MMP consistent with its inhibitory activity. These results suggest that, with the exception of TIMP-4 and SB-3CT, certain synthetic MMPIs and Δ-TIMP-2, which inhibit MT1-MMP activity and consequently autocatalytic degradation, can enhance MT1-MMP-dependent pro-MMP-2 activation in the presence of TIMP-2.

The Enhancing Effect of MMPIs on Pro-MMP-2 Activation by MT1-MMP Requires TIMP-2 for Ternary Complex Formation—To examine the relationship between the effects of the MMPIs on pro-MMP-2 activation (inhibition of MT1-MMP autocatalysis) and ternary complex formation, the *Timp2* (−/−) mutant cells were pretreated with marimastat to accumulate the 57-kDa form of MT1-MMP and then were or were not exposed to TIMP-2, Δ-TIMP-2, or TIMP-1. As expected, marimastat pretreatment and the addition of TIMP-2 (Fig. 5, *lane 2*) resulted in a significant increase in pro-MMP-2 activation when compared with the activation observed with TIMP-2 alone (*lane 1*). In contrast, administration of either Δ-TIMP-2 (*lane 3*) or TIMP-1 (*lane 4*) after the marimastat treatment had no effect. Marimastat treatment alone had no effect on pro-MMP-2 activation (*lane 5*). Immunoblot analysis demonstrated the presence of the 57-kDa form of MT1-MMP after marimastat treatment, as expected (Fig. 5, immunoblot). Taken together, these results indicate that inhibition of MT1-MMP turnover alone is not sufficient to promote pro-MMP-2 activation, a process that requires ternary complex formation. However, both processes can act synergistically to enhance activation.

Effect of Δ-TIMP-2 and Synthetic MMPIs on MT1-MMP Activity—The results above indicated a differential inhibition of MT1-MMP autocatalytic turnover by various synthetic MMPIs and Δ-TIMP-2. In order to elucidate the inhibitor effects on MT1-MMP activity observed in the cells, the interactions of the catalytic domain of MT1-MMP (MT1-MMP$_{cat}$) with natural and synthetic inhibitors were characterized in a purified system. As depicted in Table I, both TIMP-2 and Δ−TIMP-2 exhibit slow binding kinetics with similar association rate constants of

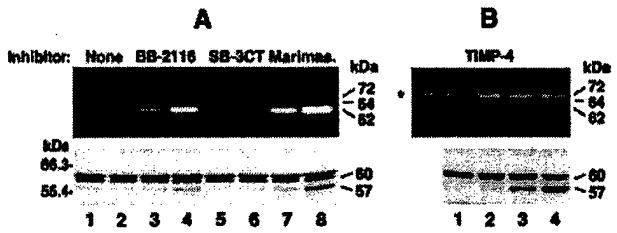

FIG. 4. **Synergistic effect of TIMP-2 with synthetic MMPIs and TIMP-4 on pro-MMP activation.** *A*, *Timp2* (−/−) mutant cells in 12-well plates were infected to express MT1-MMP as described in Fig. 1. After the infection, the media were aspirated and replaced with serum-free DMEM (1 ml/well) supplemented without (*lanes 1* and *2*) or with BB-2116 (*lane 3*, 20 nM; *lane 4*, 500 nM), SB-3CT (*lane 5*, 20 nM; *lane 6*, 500 nM) or marimastat (*lane 7*, 20 nM; *lane 8*, 500 nM). After a 16-h incubation at 37 °C, the media were aspirated, and the cells were washed with DMEM to remove excess inhibitors and then incubated with DMEM (1 ml/well) supplemented without (*lane 1*) or with (*lanes 2–8*) 10 nM TIMP-2 for 5 min at 37 °C. The media were then aspirated and replaced with DMEM containing 10 nM pro-MMP-2. After 15 min at 37 °C, the cells were solubilized in lysis buffer and analyzed for pro-MMP-2 activation and MT1-MMP forms by gelatin zymography and immunoblot analysis, respectively. *B*, *Timp2* (−/−) mutant cells in 12-well plates expressing MT1-MMP were incubated (16 h, 37 °C) without (*lane 1*) or with TIMP-4 (*lane 2*, 1 nM; *lane 3*, 10 nM; *lane 4*, 100 nM). After removal of the unbound TIMP-4, the cells received 10 nM TIMP-2 for a 5-min incubation at 37 °C followed by the addition of 10 nM pro-MMP-2 as described above. Analysis of pro-MMP-2 activation and MT1-MMP forms were monitored by gelatin zymography and immunoblot analysis, respectively.

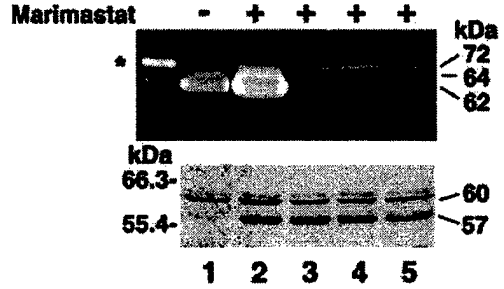

FIG. 5. **TIMP-2 is required for the enhancing effect of marimastat on pro-MMP-2 activation by MT1-MMP.** *Timp2* (−/−) mutant cells infected to express MT1-MMP in 12-well plates were treated (*lanes 2–5*) or not (*lane 1*) with 1 μM marimastat overnight at 37 °C. The medium was then aspirated and replaced with medium supplemented with either 10 nM TIMP-2 (*lanes 1* and *2*), Δ-TIMP-2 (*lane 3*), or TIMP-1 (*lane 4*) or medium without TIMPs (*lane 5*). After a 30-min incubation, the medium was aspirated, and the cells were rinsed with DMEM and incubated (30 min, 37 °C) with 10 nM pro-MMP-2. The cell lysates were analyzed for pro-MMP-2 activation and MT1-MMP forms by gelatin zymography and immunoblot analysis, respectively. The *asterisk* shows the pro-MMP-2 added to the medium.

$(2.74 \pm 0.14) \times 10^6$ and $(2.68 \pm 0.12) \times 10^6$ M^{-1} s^{-1}, respectively. The latter value is in agreement with that reported by Butler *et al.* (36) for the interaction of the (Δ128–194) TIMP-2 mutant with the catalytic domain of MT1-MMP ($2.80 \pm 0.45 \times 10^6$ M^{-1} s^{-1}). TIMP-2 bound with a picomolar K_i (0.07 nM) and showed significant inhibition at a concentration similar to that of the enzyme itself. Δ−TIMP-2 exhibits reduced affinity (K_i = 0.73 ± 0.03 nM) due to a 10-fold higher dissociation rate constant ($k_{off} = 1.95 \pm 0.03 \times 10^{-3}$ s^{-1}) relative to the value for the full-length TIMP-2. The synthetic MMP inhibitors marimastat, batimastat, BB-2116, and SB-3CT show competitive inhibition and, with the exception of SB-3CT, exhibit K_i values in the low nanomolar range. These data are in agreement with the IC_{50} values for marimastat and batimastat reported by Yamamoto *et al.* (70) for a mutant MT1-MMP lacking the transmembrane domain. In addition, the K_i value for marimastat with the $MT1\text{-}MMP_{cat}$ compares with IC_{50} values reported for the interaction of this inhibitor with the gelatinases (IC_{50} = 3–6 nM), fibroblast collagenase (IC_{50} = 5 nM), and matrylisin (IC_{50} = 16 nM) consistent with marimastat being a nonspecific (*i.e.* broad-spectrum) MMP inhibitor (33, 45, 47, 71). Interestingly, SB-3CT shows an ~10–1600-fold reduced affinity (K_i = 110 nM) for $MT1\text{-}MMP_{cat}$ compared with the other MMPIs, in agreement with its inability to induce accumulation of the 57-kDa species of MT1-MMP and pro-MMP-2 activation with TIMP-2.

Synergistic Effects of MMPI Inhibitors on Pro-MMP-2 Activation in a Background of Endogenous Expression of TIMP-2—To further examine the synergistic effects of MMPIs and TIMP-2 on pro-MMP-2 activation, we used BS-C-1 cells infected to express MT1-MMP. BS-C-1 cells produce low levels of endogenous TIMP-2, which are further suppressed but not completely eliminated upon viral infection (data not shown). Consistently, BS-C-1 cells infected to express MT1-MMP can activate pro-MMP-2 without the addition of exogenous TIMP-2 (40). Thus, we used BS-C-1 cells to examine the effects of MMPIs on pro-MMP-2 activation in a cellular system expressing a background level of endogenous TIMP-2. BS-C-1 cells infected to express MT1-MMP were incubated with increasing concentrations of TIMP-2 (0–20 nM), Δ-TIMP-2 (0–500 nM), SB-3CT (0–1 μM), or marimastat (0–10 μM) followed by the addition of pro-MMP-2. In addition, we tested the effects of Ala + TIMP-2 (0–100 nM), a mutant TIMP-2 devoid of inhibitory activity, as a negative control inhibition of MT1-MMP autocatalytic turnover. As shown in Fig. 6, pro-MMP-2 activation is greatly enhanced after administration of exogenous TIMP-2 (*TIMP-2 panel*). Both marimastat and Δ-TIMP-2 enhance pro-MMP-2 activation when compared with the basal activation detected in BS-C-1 cells in the absence of inhibitors (due to endogenous TIMP-2). Consistently, activation under these conditions is associated with accumulation of the 57-kDa species of MT1-MMP as shown in the immunoblots of Fig. 6. Both Ala + TIMP-2 and SB-3CT have no significant effects, suggesting that inhibition of the MT1-MMP autocatalytic turnover is required for the synergistic effect of the synthetic and natural MMPIs with the endogenous TIMP-2. Indeed, neither Ala + TIMP-2 nor SB-3CT induces a detectable accumulation of the 57-kDa form (Fig. 6, immunoblot). Taken together, these studies indicate that inhibition of MT1-MMP turnover (accumulation of 57-kDa form) by synthetic MMPIs can enhance the effect of the endogenous TIMP-2 in pro-MMP-2 activation in the BS-C-1 cell system.

DISCUSSION

The studies presented here provide conclusive evidence for the complex regulation of MT1-MMP activity by TIMP-2 and further demonstrate that some synthetic MMPIs might have the potential to promote MT1-MMP-dependent activation of pro-MMP-2. Our results clearly show that pro-MMP-2 activation requires the presence of TIMP-2, since the *Timp2*-null cells are unable to activate pro-MMP-2 even after expression of MT1-MMP. However, a short (5-min) incubation with exogenous TIMP-2 and a brief incubation (15 min) with pro-MMP-2 result in a significant conversion of pro-MMP-2 to its active form. This rapid activation of pro-MMP-2 is unprecedented in a cellular system and demonstrates the high catalytic efficiency of MT1-MMP for this substrate under optimal conditions. The dependence on TIMP-2 for activation is also evident from the results with the heterozygous *Timp2* (+/−) mutant cells and the BS-C-1 cells, both of which contain endogenous TIMP-2 and are able to activate pro-MMP-2 after expression of MT1-MMP without requirement of exogenous TIMP-2. Strongin *et al.* (12) proposed that the effect of TIMP-2 on activation is mediated by a ternary complex formed between active MT1-MMP, TIMP-2, and pro-MMP-2, where the C-terminal region of TIMP-2 binds

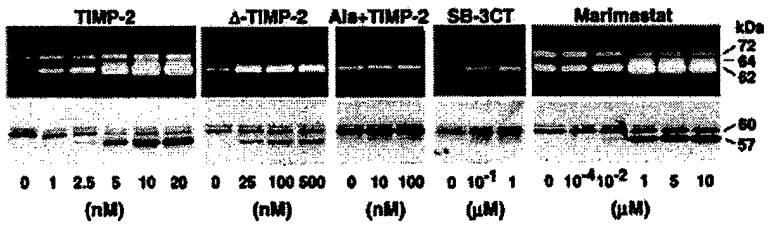

FIG. 6. **Effect of synthetic MMPIs on pro-MMP-2 activation in BS-C-1 cells expressing MT1-MMP and endogenous TIMP-2.** BS-C-1 cells were co-infected to express MT1-MMP as described previously (40). After infection, the media were aspirated and replaced with serum-free DMEM supplemented with various concentrations of TIMP-2, marimastat, Δ-TIMP-2, Ala + TIMP-2, or SB-3CT for a 16-h incubation at 37 °C. The media were then aspirated and replaced with DMEM containing 10 nM pro-MMP-2. After a 30-min incubation at 37 °C, the cells were rinsed with phosphate-buffered saline and then lysed in lysis buffer. The lysates were subjected to gelatin zymography and immunoblot analysis for assessment of pro-MMP-2 activation and generation of the 57-kDa form of MT1-MMP, respectively.

to pro-MMP-2 to anchor the zymogen to the cell surface. Although formation of such a complex was not directly demonstrated in the present study, the results with the Δ-TIMP-2, which lacks the C-terminal domain and failed to induce pro-MMP-2 activation in the *Timp2* (−/−) mutant cells, indirectly support the ternary complex model of pro-MMP-2 activation.

In addition to its role in ternary complex formation, TIMP-2 also influences the processing of MT1-MMP. We have recently shown that TIMP-2 prevents the autocatalytic conversion of active MT1-MMP (57 kDa) to its inactive 44-kDa species, and as a consequence the 57-kDa species accumulates on the cell surface (40). As shown here, a similar effect is induced by synthetic MMPIs (41) as well as by Δ-TIMP-2 and TIMP-4. We show for the first time that some synthetic MMPIs and Δ-TIMP-2 can enhance pro-MMP-2 activation by MT1-MMP in the presence of TIMP-2. This effect is due to the accumulation of the 57-kDa MT1-MMP species as a consequence of inhibition of MT1-MMP turnover. Our kinetic data suggest the possibility that the increase in MT1-MMP·TIMP-2 complexes may be a consequence of a displacement of the bound synthetic MMPI by TIMP-2 (K_i for TIMP-2 is approximately 1–2 orders of magnitude lower than that for the synthetic MMPI), a process that will generate more pro-MMP-2 "receptors." However, binding of small molecule inhibitors concurrently with TIMP-2 to active MT1-MMP cannot be ruled out. Regardless of the mechanism involved, pro-MMP-2 activation would require a "catalytic" quantity of the inhibitor-free MT1-MMP to hydrolyze the Asn^{37}-Leu^{38} bond of pro-MMP-2 as previously shown (69). It should be noted that the enhancing effects of the MMPIs on pro-MMP-2 activation in the *Timp2* null cells in the presence of TIMP-2 were evident only when the synthetic inhibitors (up to 10 μM to avoid toxic effects) were administered to the cells and removed from the system prior to the administration of the exogenous TIMP-2. Preincubation of the cells with the MMPIs was necessary to induce accumulation of the 57-kDa form of MT1-MMP, a fraction of which would be expected to be inhibitor-free, since the cells are continuously producing MT1-MMP. We postulate that these conditions (removal of excess synthetic inhibitor prior to the addition of TIMP-2 and pro-MMP-2 and continuous replenishment of MT1-MMP by the cells) generate sufficient catalytically active MT1-MMP to generate ternary complex and to process pro-MMP-2. In contrast, simultaneous administration of TIMP-2 with various doses of marimastat inhibits pro-MMP-2 activation in a dose-dependent manner (data not shown). This inhibitory effect is likely to be due to competition between TIMP-2 and marimastat for MT1-MMP binding, resulting in decreased ternary complex formation (38, 72). However, BS-C-1 cells, which contain low levels of endogenous TIMP-2, exhibit enhanced pro-MMP-2 activation upon administration of the MMPIs. In this case, enhanced activation is the result of the inhibition of MT1-MMP autocatalysis and ternary complex formation is not a limiting step. The result with the BS-C-1 cells also suggests that invasive tumor cells equipped with both MT1-MMP and TIMP-2 may be subject to similar synergistic effects of synthetic MMPIs on MT1-MMP activity under the right conditions.

The relationship between inhibition of MT1-MMP autocatalysis and ternary complex formation was also demonstrated in the experiments in which the *Timp2* (−/−) mutant cells were treated with marimastat followed by administration of Δ-TIMP-2 or TIMP-1, in which case pro-MMP-2 activation was not observed. Furthermore, the Ala + TIMP-2 mutant, devoid of inhibitory activity, failed to support pro-MMP-2 activation in the BS-C-1 cells in the presence of endogenous TIMP-2 due to its inability to inhibit MT1-MMP turnover. Likewise, pretreatment of the *Timp2* (−/−) cells with marimastat had no effect on pro-MMP-2 activation without subsequent addition of TIMP-2 revealing that accumulation of active MT1-MMP alone is not sufficient for pro-MMP-2 activation and requires a functional full-length TIMP-2 to generate the ternary complex. This was also demonstrated by the results with TIMP-4, which, despite its ability to inhibit MT1-MMP activity as found by Bigg *et al.*[2] and to bind pro-MMP-2 (68), was unable to promote MT1-MMP-dependent activation of pro-MMP-2 or to act synergistically with TIMP-2 in this process. Recent studies from Overall's laboratory[2] also show that TIMP-4 cannot form a ternary complex in a purified system and, if administered with TIMP-2, inhibits pro-MMP-2 activation by MT1-MMP in *Timp2* mutant cells. Here we have shown that TIMP-4, like other MMPIs, induces accumulation of the 57-kDa form of MT1-MMP, consistent with its inhibitory activity, but fails to act synergistically with TIMP-2 in the promotion of pro-MMP-2 activation. The reason for this puzzling result is yet unknown but may be related to differences in affinity between these inhibitors for MT1-MMP and pro-MMP-2. Indeed, TIMP-4 exhibits a lower affinity for pro-MMP-2 when compared with TIMP-2.[2] Also, a potential rapid internalization of the putative MT1-MMP·TIMP-4 complex, although yet unproven, may play a role. Further enzymatic and biochemical studies are required to understand the dynamics of TIMP-4 and TIMP-2 inhibitory activities in relation to MT1-MMP functions. Nevertheless, these studies suggest that the effects of TIMP-4 on TIMP-2 may represent a natural and unique regulatory mechanism of MMP-dependent proteolysis on the cell surface in which TIMP-4 may play a counter role to that of TIMP-2, physiologically, by binding to active MT1-MMP with high affinity. We therefore propose that the long held view of a balance between MMPs and TIMPs as a key determinant of proteolytic activity and tumor progression (73) may well include a balance of TIMP-2 and TIMP-4 as a major determining factor for MT1-MMP-dependent proteolysis in cancer tissues where both inhibitors may be present.

The results presented here demonstrate that pro-MMP-2 activation by MT1-MMP at the cell surface is the result of a

highly regulated enzymatic process that involves two independent events, which under certain conditions may work synergistically to enhance MT1-MMP-dependent activation of pro-MMP-2. It should be noted that this might not be the case in all circumstances or with different MT1-MMP substrates. For example, for pro-MMP-2, our data show that a short (5-min) exposure to TIMP-2 followed by a 15-min incubation with pro-MMP-2 was sufficient to rapidly activate pro-MMP-2 without detectable accumulation of active (57-kDa) MT1-MMP. The reason for the lack of detection of active enzyme under this conditions is unclear but may be related to the detection method (immunoblotting), rapid enzyme turnover, and/or the internalization and turnover of the MT1-MMP (57 kDa)·TIMP-2 complex as recently reported (72). Under conditions of substoichiometric TIMP-2 molar concentrations relative to MT1-MMP, the efficient binding of TIMP-2 and the catalytic efficiency of MT1-MMP for its substrate result in optimal pro-MMP-2 activation (39). Thus, while rapid bursts of TIMP-2 expression will be sufficient to generate ternary complex and consequently activate pro-MMP-2 in the absence of a significant and detectable accumulation of active MT1-MMP, chronic exposure to TIMP-2 or MMPIs would maintain a steady level of MT1-MMP on the cell surface due to inhibition of autocatalysis. For other MT1-MMP substrates such as ECM components, which do not require ternary complex formation to be hydrolyzed by MT1-MMP, sustained TIMP-2 expression and/or the presence of synthetic MMPIs may indirectly enhance catalytic activity, as demonstrated here using pro-MMP-2 as a target substrate.

Recent accomplishments in drug design have resulted in the generation of a variety of novel MMPIs with effective antitumor and anti-angiogenic activities in animal models of cancer (3, 4, 33, 46, 47). These encouraging results have brought some of these compounds, such as marimastat and batimastat, to human clinical trials. The majority of the compounds undergoing testing in humans, however, lack specificity toward the various MMP families. The hydroxamates, for instance, inhibit a wide spectrum of MMPs, including MT1-MMP as herein demonstrated, often with similar affinities (70, 71). The complex outcome of MT1-MMP inhibition on catalytic activity demonstrated here raises important issues regarding the potential consequences of inhibiting MT1-MMP. The intermolecular autocatalytic turnover of MT1-MMP on the cell surface may represent an important regulatory step aimed at controlling pericellular proteolysis, a process that is likely to be favored by lateral diffusion and clustering of MT1-MMP molecules in the cell surface (74, 75). Thus, reversible inhibition of MT1-MMP activity would play a role in preventing excessive enzyme clearance from the cell surface and indirectly favor proteolysis. Such an effect by synthetic MMPIs would depend on the spectrum of activity (K_i values) elicited by each particular inhibitor against the different members of the MMP family and on their pharmacokinetics and dosing regime. The MMPIs tested here exhibit different K_i values for the catalytic domain of MT1-MMP, which correlated well with their efficacy in promoting pro-MMP-2 activation with TIMP-2. We recently described the first example of a mechanism-based inhibitor for MMPs (42). This inhibitor, SB-3CT, is highly specific for inhibition of gelatinases, enzymes that were inhibited covalently by this inhibitor. SB-3CT does not pursue the metal chelation strategy for its inhibition, in contrast to the case of the existing inhibitors. We have shown here that SB-3CT is substantially less effective in inhibition of MT1-MMP, for which it was not designed, and simply behaves as a simple linear competitive inhibitor, in contrast to the case of gelatinases (42). Again in contrast to marimastat and batimastat, SB-3CT did not show any ability to stimulate activation of pro-MMP-2 induced by TIMP-2. Thus, the design of highly specific MMPIs will minimize potential adverse effects in conditions where inhibition of the MT1-MMP-MMP-2 system is a therapeutic goal.

The complex regulation of MT1-MMP activity by TIMP-2 may provide a biochemical framework for understanding several intriguing observations in human tumors and experimental models of metastasis using synthetic MMPIs. High levels of TIMP-2 expression were found in various human cancers, which positively correlated with metastasis and poor survival (21, 76–79). A recent study reported that treatment of tumor-bearing mice with batimastat significantly inhibited tumor growth but promoted tumor cell invasion into the liver of a variety of human cancer cells (80). However, the mechanism for such effect was not reported. Finally, recent tumorigenicity studies with the heterozygous and homozygous *Timp2* mutant cells indicate a higher incidence of tumor formation and metastasis in the heterozygous cells, suggesting a role for TIMP-2 in promotion of tumor progression.[5] Our findings disclosed in this report provide one plausible explanation for these observations, that by binding to active MT1-MMP, both natural and synthetic MMP inhibitors may produce a "pool" of active MT1-MMP available to degrade ECM components and to activate pro-MMP-2. While this may represent an undesired effect of some strategies for anti-MMP therapies in cancer that are being investigated, this effect may be beneficial in pathological conditions characterized by excessive deposition of collagen such as fibrosis and connective tissue disorders where increased MMP activity might be desired. These examples and the studies presented herein emphasize the importance of a rational approach for the design of specific MMP inhibitor, which should also be based on an understanding of the regulation of MT1-MMP and likely other members of the MT-MMP subfamily by TIMPs and MMPIs at the cell surface.

REFERENCES

1. Nagase, H., and Woessner, J. F., Jr. (1999) *J. Biol. Chem.* **274,** 21491–21494
2. Birkedal-Hansen, H., Moore, W. G., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, B., DeCarlo, A., and Engler, J. A. (1993) *Crit. Rev. Oral Biol. Med.* **4,** 197–250
3. Stetler-Stevenson, W. G. (1999) *J. Clin. Invest.* **103,** 1237–1241
4. Nelson, A. R., Fingleton, B., Rothenberg, M. L., and Matrisian, L. M. (2000) *J. Clin. Oncol.* **18,** 1135–1149
5. Massova, I., Kotra, L. P., Fridman, R., and Mobashery, S. (1998) *FASEB J.* **12,** 1075–1095
6. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. (1994) *Nature* **370,** 61–65
7. Puente, X. S., Pendas, A. M., Llano, E., Velasco, G., and Lopez-Otin, C. (1996) *Cancer Res.* **56,** 944–949
8. Pei, D. (1999) *J. Biol. Chem.* **274,** 8925–8932
9. Pei, D. (1999) *Cell Res.* **9,** 291–303
10. Velasco, G., Cal, S., Merlos-Suarez, A., Ferrando, A. A., Alvarez, S., Nakano, A., Arribas, J., and Lopez-Otin, C. (2000) *Cancer Res.* **60,** 877–882
11. Takino, T., Sato, H., Shinagawa, A., and Seiki, M. (1995) *J. Biol. Chem.* **270,** 23013–23020
12. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I. (1995) *J. Biol. Chem.* **270,** 5331–5338
13. Pei, D., and Weiss, S. J. (1996) *J. Biol. Chem.* **271,** 9135–9140
14. d'Ortho, M. P., Stanton, H., Butler, M., Atkinson, S. J., Murphy, G., and Hembry, R. M. (1998) *FEBS Lett.* **421,** 159–164
15. d'Ortho, M. P., Will, H., Atkinson, S., Butler, G., Messent, A., Gavrilovic, J., Smith, B., Timpl, R., Zardi, L., and Murphy, G. (1997) *Eur. J. Biochem.* **250,** 751–757
16. Ohuchi, E., Imai, K., Fujii, Y., Sato, H., Seiki, M., and Okada, Y. (1997) *J. Biol. Chem.* **272,** 2446–2451
17. Zhou, Z., Apte, S. S., Soininen, R., Cao, R., Baaklini, G. Y., Rauser, R. W., Wang, J., Cao, Y., and Tryggvason, K. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 4052–4057
18. Holmbeck, K., Bianco, P., Caterina, J., Yamada, S., Kromer, M., Kuznetsov, S. A., Mankani, M., Robey, P. G., Poole, A. R., Pidoux, I., Ward, J. M., and Birkedal-Hansen, H. (1999) *Cell* **99,** 81–92
19. Kleiner, D. E., and Stetler-Stevenson, W. G. (1999) *Cancer Chemother. Pharmacol.* **43,** (suppl.) 42–51
20. Jones, J. L., Glynn, P., and Walker, R. A. (1999) *J. Pathol.* **189,** 161–168
21. Davidson, B., Goldberg, I., Kopolovic, J., Lerner-Geva, L., Gotlieb, W. H., Ben-Baruch, G., and Reich, R. (1999) *Gynecol. Oncol.* **73,** 372–382

[5] P. Soloway, manuscript in preparation.

22. Kitagawa, Y., Kunimi, K., Ito, H., Sato, H., Uchibayashi, T., Okada, Y., Seiki, M., and Namiki, M. (1998) *J. Urol.* **160,** 1540–1545
23. Kurahara, S., Shinohara, M., Ikebe, T., Nakamura, S., Beppu, M., Hiraki, A., Takeuchi, H., and Shirasuna, K. (1999) *Head Neck* **21,** 627–638
24. Ueno, H., Nakamura, H., Inoue, M., Imai, K., Noguchi, M., Sato, H., Seiki, M., and Okada, Y. (1997) *Cancer Res.* **57,** 2055–2060
25. Yoshizaki, T., Sato, H., Maruyama, Y., Murono, S., Furukawa, M., Park, C. S., and Seiki, M. (1997) *Cancer* **79,** 139–144
26. Harada, T., Arii, S., Mise, M., Imamura, T., Higashitsuji, H., Furutani, M., Niwano, M., Ishigami, S., Fukumoto, M., Seiki, M., Sato, H., and Imamura, M. (1998) *J. Hepatol.* **28,** 231–239
27. Hiraoka, N., Allen, E., Apel, I. J., Gyetko, M. R., and Weiss, S. J. (1998) *Cell* **95,** 365–377
28. Rosenthal, E. L., Hotary, K., Bradford, C., and Weiss, S. J. (1999) *Otolaryngol. Head Neck Surg.* **121,** 337–343
29. Tsunezuka, Y., Kinoh, H., Takino, T., Watanabe, Y., Okada, Y., Shinagawa, A., Sato, H., and Seiki, M. (1996) *Cancer Res.* **56,** 5678–5683
30. Kadono, Y., Okada, Y., Namiki, M., Seiki, M., and Sato, H. (1998) *Cancer Res.* **58,** 2240–2244
31. Hotary, K., Allen, E., Punturieri, A., Yana, I., and Weiss, S. J. (2000) *J. Cell Biol.* **149,** 1309–1323
32. Yu, A. E., Hewitt, R. E., Connor, E. W., and Stetler-Stevenson, W. G. (1997) *Drugs Aging* **11,** 229–244
33. Rasmussen, H. S., and McCann, P. P. (1997) *Pharmacol. Ther.* **75,** 69–75
34. Overall, C. M., King, A. E., Bigg, H. F., McQuibban, A., Atherstone, J., Sam, D. K., Ong, A. D., Lau, T. T., Wallon, U. M., DeClerck, Y. A., and Tam, E. (1999) *Ann. N. Y. Acad. Sci.* **878,** 747–753
35. Fridman, R., Fuerst, T. R., Bird, R. E., Hoyhtya, M., Oelkuct, M., Kraus, S., Komarek, D., Liotta, L. A., Berman, M. L., and Stetler-Stevenson, W. G. (1992) *J. Biol. Chem.* **267,** 15398–15405
36. Butler, G. S., Butler, M. J., Atkinson, S. J., Will, H., Tamura, T., van Westrum, S. S., Crabbe, T., Clements, J., d'Ortho, M. P., and Murphy, G. (1998) *J. Biol. Chem.* **273,** 871–880
37. Fernandez-Catalan, C., Bode, W., Huber, R., Turk, D., Calvete, J. J., Lichte, A., Tschesche, H., and Maskos, K. (1998) *EMBO J.* **17,** 5238–5248
38. Zucker, S., Drews, M., Conner, C., Foda, H. D., DeClerck, Y. A., Langley, K. E., Bahou, W. F., Docherty, A. J., and Cao, J. (1998) *J. Biol. Chem.* **273,** 1216–1222
39. Jo, Y., Yeon, J., Kim, H. J., and Lee, S. T. (2000) *Biochem. J.* **345,** 511–519
40. Hernandez-Barrantes, S., Toth, M., Bernardo, M. M., Yurkova, M., Gervasi, D. C., Raz, Y., Sang, Q. A., and Fridman, R. (2000) *J. Biol. Chem.* **275,** 12080–12089
41. Stanton, H., Gavrilovic, J., Atkinson, S. J., d'Ortho, M. P., Yamada, K. M., Zardi, L., and Murphy, G. (1998) *J. Cell Sci.* **111,** 2789–2798
42. Brown, S., Bernardo, M. M., Zhi-Hong, L., Kotra, L. P., Tanaka, Y., Fridman, R., and Mobashery, S. (2000) *J. Am. Chem. Soc.* **122,** 6799–6780
43. Belotti, D., Paganoni, P., and Giavazzi, R. (1999) *Int. J. Biol. Markers* **14,** 232–238
44. Wojtowicz-Praga, S. (1999) *Drugs Res. Dev.* **1,** 117–129
45. Steward, W. P. (1999) *Cancer Chemother. Pharmacol.* **43,** (suppl.) 56–60
46. De, B., Natchus, M. G., Cheng, M., Pikul, S., Almstead, N. G., Taiwo, Y. O., Snider, C. E., Chen, L., Barnett, B., Gu, F., and Dowty, M. (1999) *Ann. N. Y. Acad. Sci.* **878,** 40–60
47. Brown, P. D. (1997) *Med. Oncol.* **14,** 1–10
48. Soloway, P. D., Alexander, C. M., Werb, Z., and Jaenisch, R. (1996) *Oncogene* **13,** 2307–2314
49. Mortensen, R. M., Conner, D. A., Chao, S., Geisterfer-Lowrance, A. A., and Seidman, J. G. (1992) *Mol. Cell. Biol.* **12,** 2391–2395
50. Fuerst, T. R., Earl, P. L., and Moss, B. (1987) *Mol. Cell. Biol.* **7,** 2538–2544
51. Fridman, R., Bird, R. E., Hoyhtya, M., Oelkuct, M., Komarek, D., Liang, C. M., Berman, M. L., Liotta, L. A., Stetler-Stevenson, W. G., and Fuerst, T. R. (1993) *Biochem. J.* **289,** 411–416
52. Olson, M. W., Gervasi, D. C., Mobashery, S., and Fridman, R. (1997) *J. Biol. Chem.* **272,** 29975–29983
53. Ko, Y. C., Langley, K. E., Mendiaz, E. A., Parker, V. P., Taylor, S. M., and DeClerck, Y. A. (1997) *Biochem. Cell Biol. Commun.* **236,** 100–105
54. Wingfield, P. T., Sax, J. K., Stahl, S. J., Kaufman, J., Palmer, I., Chung, V., Corcoran, M. L., Kleiner, D. E., and Stetler-Stevenson, W. G. (1999) *J. Biol. Chem.* **274,** 21362–21368
55. Lichte, A., Kolkenbrock, H., and Tschesche, H. (1996) *FEBS Lett.* **397,** 277–282
56. Olson, M. W., Bernardo, M. M., Pietila, M., Gervasi, D. C., Toth, M., Kotra, L. P., Massova, I., Mobashery, S., and Fridman, R. (2000) *J. Biol. Chem.* **275,** 2661–2668
57. Hoyhtya, M., Fridman, R., Komarek, D., Porter-Jordan, K., Stetler-Stevenson, W. G., Liotta, L. A., and Liang, C. M. (1994) *Int. J. Cancer* **56,** 500–505
58. Gervasi, D. C., Raz, A., Dehem, M., Yang, M., Kurkinen, M., and Fridman, R. (1996) *Biochem. Cell Biol. Commun.* **228,** 530–538
59. Li, H., Bauzon, D. E., Xu, X., Tschesche, H., Cao, J., and Sang, Q. A. (1998) *Mol. Carcinog.* **22,** 84–94
60. Laemmli, U. K. (1970) *Nature* **227,** 680–685
61. Toth, M., Gervasi, D. C., and Fridman, R. (1997) *Cancer Res.* **57,** 3159–3167
62. Knight, C. G. (1995) *Methods Enzymol.* **248,** 18–34
63. Muller-Steffner, H. M., Malver, O., Hosie, L., Oppenheimer, N. J., and Schuber, F. (1992) *J. Biol. Chem.* **267,** 9606–9611
64. Segel, I. H. (1993) *Enzyme Kinetics*, pp. 100–160 John Wiley & Sons, Inc., New York
65. Wang, Z., Juttermann, R., and Soloway, P. D. (2000) *J. Biol. Chem.* **275,** 26411–26415
66. Overall, C. M., and Sodek, J. (1990) *J. Biol. Chem.* **265,** 21141–21151
67. Caterina, J. J., Yamada, S., Caterina, N. C., Longenecker, G., Holmbäck, K., Shi, J., Yermovsky, A. E., Engler, J. A., and Birkedal-Hansen, H. (2000) *J. Biol. Chem.* **275,** 26416–26422
68. Bigg, H. F., Shi, Y. E., Liu, Y. E., Steffensen, B., and Overall, C. M. (1997) *J. Biol. Chem.* **272,** 15496–15500
69. Will, H., Atkinson, S. J., Butler, G. S., Smith, B., and Murphy, G. (1996) *J. Biol. Chem.* **271,** 17119–17123
70. Yamamoto, M., Tsujishita, H., Hori, N., Ohishi, Y., Inoue, S., Ikeda, S., and Okada, Y. (1998) *J. Med. Chem.* **41,** 1209–1217
71. Brown, P. D. (1999) *Apmis* **107,** 174–180
72. Maquoi, E., Frankenne, F., Baramova, E., Munaut, C., Sounni, N. E., Remacle, A., Noel, A., Murphy, G., and Foidart, J. M. (2000) *J. Biol. Chem.* **275,** 11368–11378
73. Liotta, L. A., Steeg, P. S., and Stetler-Stevenson, W. G. (1991) *Cell* **64,** 327–336
74. Nakahara, H., Howard, L., Thompson, E. W., Sato, H., Seiki, M., Yeh, Y., and Chen, W. T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 7959–7964
75. Nabeshima, K., Inoue, T., Shimao, Y., Okada, Y., Itoh, Y., Seiki, M., and Koono, M. (2000) *Cancer Res.* **60,** 3364–3369
76. Kanayama, H., Yokota, K., Kurokawa, Y., Murakami, Y., Nishitani, M., and Kagawa, S. (1998) *Cancer* **82,** 1359–1366
77. Murashige, M., Miyahara, M., Shiraishi, N., Saito, T., Kohno, K., and Kobayashi, M. (1996) *Jpn. J. Clin. Oncol.* **26,** 303–309
78. Ree, A. H., Florenes, V. A., Berg, J. P., Maelandsmo, G. M., Nesland, J. M., and Fodstad, O. (1997) *Clin. Cancer Res.* **3,** 1623–1628
79. Visscher, D. W., Hoyhtya, M., Ottosen, S. K., Liang, C. M., Sarkar, F. H., Crissman, J. D., and Fridman, R. (1994) *Int. J. Cancer* **59,** 339–344
80. Della Porta, P., Soeltl, R., Krell, H. W., Collins, K., O'Donoghue, M., Schmitt, M., and Kruger, A. (1999) *Anticancer Res.* **19,** 3809–3816

Biochemical and Biophysical Research Communications **281**, 126–130 (2001)
doi:10.1006/bbrc.2001.4323, available online at http://www.idealibrary.com on IDEAL®

Differential Roles of TIMP-4 and TIMP-2 in Pro-MMP-2 Activation by MT1-MMP

Sonia Hernandez-Barrantes,* Yoichiro Shimura,* Paul D. Soloway,†
QingXiang Amy Sang,‡ and Rafael Fridman*,[1]

*Department of Pathology, School of Medicine, Wayne State University, Detroit, Michigan 48201; †Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York 14263; and ‡Department of Chemistry, Biochemistry Division, Florida State University, Tallahassee, Florida 32306

Received January 9, 2001

The tissue inhibitors of metalloproteinases (TIMPs) are specific inhibitors of MMP enzymatic activity. However, TIMP-2 can promote the activation of pro-MMP-2 by MT1-MMP. This process is mediated by the formation of a complex between MT1-MMP, TIMP-2, and pro-MMP-2. Binding of TIMP-2 to active MT1-MMP also inhibits the autocatalytic turnover of MT1-MMP on the cell surface. Thus, under certain conditions, TIMP-2 is a positive regulator of MMP activity. TIMP-4, a close homologue of TIMP-2 also binds to pro-MMP-2 and can potentially participate in pro-MMP-2 activation. We coexpressed MT1-MMP with TIMP-4 and investigated its ability to support pro-MMP-2 activation. TIMP-4, unlike TIMP-2, does not promote pro-MMP-2 activation by MT1-MMP. However, TIMP-4 binds to MT1-MMP inhibiting its autocatalytic processing. When coexpressed with TIMP-2, TIMP-4 competitively reduced pro-MMP-2 activation by MT1-MMP. A balance between TIMP-2 and TIMP-4 may be a critical factor in determining the degradative potential of cells in normal and pathological conditions. © 2001 Academic Press

Key Words: **matrix metalloproteinases; TIMP; proteases; membrane proteins; cell surface.**

The activation of the zymogenic form of MMP-2 (pro-MMP-2) has been shown to be accomplished by the membrane-tethered MT1-MMP (MMP-14) (1, 2), which hydrolyses the Asn^{37}-Leu^{38} peptide bond in the prodomain of pro-MMP-2 (3). To facilitate the association of the prodomain of pro-MMP-2 with the active site of MT1-MMP, pro-MMP-2 must be positioned in close association with MT1-MMP. To achieve this, TIMP-2 acts as a molecular link between pro-MMP-2 and MT1-MMP (1). It has been shown that the NH_2-terminal region of TIMP-2 binds to the active site of an active MT1-MMP on the cell surface generating a pro-MMP-2 "receptor" (4). In turn, the COOH-terminal region of TIMP-2 binds to the COOH-terminal domain of pro-MMP-2, also known as the hemopexin-like domain (HLD) (1, 4–8). This trimeric MT1-MMP/TIMP-2/pro-MMP-2 complex permits the association of pro-MMP-2 to the cell surface, which eventually facilitates its activation by a neighboring TMP-2-free MT1-MMP. Under these conditions, TIMP-2 acts as a positive regulator of pro-MMP-2 activation (9). We have recently shown that in addition to its ability to form the pro-MMP-2 receptor, TIMP-2 can also regulate the nature of MT1-MMP forms present in the cells by its ability to inhibit MT1-MMP activity (6). This effect is due to the TIMP-2 inhibition of the autocatalytic turnover of MT1-MMP on the cell surface, which may represent a natural mechanism of clearance of active MT1-MMP from the cell surface once the enzyme has fulfilled its pericellular proteolytic function. As a consequence of the inhibition of autocatalytic degradation, *de novo* synthesis and membrane incorporation of new MT1-MMP molecules, TIMP-2 binding to active MT1-MMP results in accumulation of active MT1-MMP on the cell surface (6). These dual effects of TIMP-2 (ternary complex formation and inhibition of MT1-MMP autocatalytic turnover) contribute to the overall effects of MT1-MMP on the cell surface: activation of pro-MMP-2 and direct pericellular proteolysis (10, 11).

TIMP-2 belongs to a family of four TIMP inhibitors, which presently includes TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (12). Studies on TIMP-MMP interactions have shown that TIMP-4, like TIMP-2 is also capable of forming a complex with pro-MMP-2, which is mediated by binding of the inhibitor to the HLD of the enzyme (13). Thus, functionally, TIMP-4 is similar to TIMP-2. In addition, sequence analyses revealed a 70% identity between TIMP-2 and TIMP-4 (12, 14). Based on these

[1] To whom correspondence should be addressed at Department of Pathology, Wayne State University, 540 E. Canfield Ave., Detroit, MI 48201. Fax: 313-577-8180. E-mail: rfridman@med.wayne.edu.

0006-291X/01 $35.00

observations, we asked whether TIMP-4, like TIMP-2, could also promote pro-MMP-2 activation by MT1-MMP. To answer this question, we expressed human TIMP-4 in a vaccinia expression system (15, 16) and tested its ability to promote pro-MMP-2 activation and inhibition of MT1-MMP autocatalysis in mammalian cells expressing MT1-MMP. Our studies demonstrate that unlike TIMP-2, TIMP-4 cannot promote MT1-MMP dependent pro-MMP-2 activation but can inhibit MT1-MMP autocatalytic degradation. Furthermore, if co-expressed with TIMP-2, TIMP-4 reduces the rate of pro-MMP-2 activation induced by TIMP-2.

MATERIALS AND METHODS

Cells. Nonmalignant monkey kidney epithelial BS-C-1 (CCL-26) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Immortalized homozygous *Timp2* (−/−) mutant mouse fibroblasts were isolated from *Timp2* deficient mice and immortalized by retroviral infection as described (7, 17, 18) and maintained in DMEM supplemented with 10% FBS and antibiotics. All tissue culture reagents were purchased from Gibco BRL (Grand Island, NY).

Vaccinia virus and construction of expression vectors. The generation of the vaccinia expression vector pTF7EMCV-1 (pTF7) containing the T7 RNA promoter and the production of vTF7-3, a recombinant vaccinia virus expressing bacteriophage T7 RNA polymerase, have been described by Fuerst *et al.* (16). The generation of the pTF7-T2 vector expressing human TIMP-2 (15) and pTF7-MT1 expressing human MT1-MMP (6) has been described. The human full-length TIMP-4 cDNA (14), a generous gift from Dr. Y. E. Shi (Albert Einstein College of Medicine, New Hyde Park, NY 11042), was amplified by the polymerase chain reaction (PCR) using the following oligonucleotide primers: 5′-CATTCCATGGCACCTGGGAGCCCT-3′ and 5′-CTTGGATCCCTAGGGCTGAACGATGTCAAC-3′ containing the NcoI and BamHI restriction sites, respectively. The amplified TIMP-4 fragment was isolated and cloned into pTF7 vector (16) to generate the pTF7-T4 plasmid. The DNA sequence of the TIMP-4 PCR fragment was verified by sequencing of both strands directly from the pTF7-T4 vector using an ABI377A DNA sequencer.

Expression of recombinant proteins by infection–transfection. Monkey kidney BS-C-1 or *Timp2* (−/−) mutant cells in 6-well plates were infected with 30 plaque-forming units (pfu)/cell of the vTF-3 virus in DMEM containing 2.5% FBS (15). Thirty minutes postinfection, the media were aspirated and the infected cells were cotransfected with a mixture of either pTF7-MT1 and pTF7-T4 plasmids (04 μg/ml each) or pTF7-MT1 and pTF7-T2 plasmids (04 μg/ml each) to coexpress MT1-MMP with each inhibitor using Effectene (Qiagen, Valencia, CA), as described by the manufacturer. In some experiments, MT1-MMP was coexpressed with both TIMP-4 and TIMP-2 by transfecting a mixture of the three plasmids (04 μg/ml each). As controls, the infected cells were transfected with pTF7-MT1, pTF7-T4, pTF7-T2 or the empty pFT7EMCV-1 expression vectors alone. An additional control included cells infected with vTF-3 virus but nontransfected. Four h post-transfection, the media were aspirated and replaced with 1 ml/well of OPTI-MEM (Gibco BRL).

Activation of pro-MMP-2. Eighteen hours following the infection/transfection procedure, the cells received 3 nM/well of purified recombinant pro-MMP-2, purified to homogeneity as previously described (15), followed by a 4-h incubation at 37°C. The media were collected and clarified by a brief centrifugation (13,000*g*, 15 min, 4°C) and the cells were solubilized in 100 μl/well of cold lysis buffer (25 mM Tris–HCl [pH 7.5], 1% IGEPAL CA-630, 100 mM NaCl, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM benzamidine, and 1 mM PMSF) and centrifuged (13,000*g*) for 15 min at 4°C. Samples of the lysates (10 μl) were mixed with 4X Laemmli sample buffer (19) without reducing agents and without heating and subjected to gelatin zymography, as previously described (20).

Immunoprecipitation of TIMP-2 and TIMP-4. BS-C-1 cells Infected-transfected to express TIMP-2 or TIMP-4 with or without MT1-MMP, in 6-well plates, were metabolically labeled for 4 h at 37°C with 100 μCi/ml of ^{35}S-methionine in 1 ml/well of DMEM without methionine supplemented with 1% dialyzed FBS. The media were collected, clarified by a brief centrifugation and an aliquot incubated (16 h, 4°C) with either 5 μg of anti-TIMP-2 CA-101 monoclonal antibody or a rabbit polyclonal antibody to TIMP-4 (21) followed by the addition of 30 μl of Protein G-Sepharose beads for an additional 3-h incubation at 4°C. After recovering the beads by a brief centrifugation, the beads were washed (5 times) with cold 50 mM Tris–HCl pH 7.5 containing 150 mM NaCl, 0.1% NP-40, and 10% glycerol and resuspended in 15 μl Laemmli sample buffer with β-mercaptoethanol followed by boiling (5 min). The immunoprecipitates were resolved by 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Detection of radiolabeled proteins was performed by autoradiography.

Immunoblot analysis of MT1-MMP forms. Infected BS-C-1 cells in 6-well plates, as described above, were lysed in cold lysis buffer. The lysates were mixed with 4× Laemmli sample buffer with β-mercaptoethanol and then resolved by 10% SDS–PAGE followed by transfer to a nitrocellulose membrane as described (20). Detection of MT1-MMP and TIMP-4 were carried out using a polyclonal antibody to human MT1-MMP (pAb 437) (22) and a polyclonal antibody to human TIMP-4 (21), respectively. Detection of the antigen-antibody complex was performed using the SuperSignal Enhanced Chemiluminescence (ECL) system (Pierce, Rockford, IL), according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Our previous studies demonstrated that co-expression of human MT1-MMP with TIMP-2 in a vaccinia expression system resulted in enhanced activation of pro-MMP-2 (6). Activation of pro-MMP-2 by MT1-MMP was TIMP-2-dependent since no activation was observed in cells devoid of TIMP-2 (*Timp2* mutant cells) (7). Furthermore, TIMP-2 presence induced the accumulation of the 57-kDa form of MT1-MMP, which represents the active enzyme starting at Y^{112}. Concomitantly with the accumulation of the 57-kDa form there was a reduction of the 44–40-kDa autocatalytic product of MT1-MMP in the cell lysates (7). To examine the effects of TIMP-4 on pro-MMP-2 activation, the full-length human TIMP-4 cDNA was cloned into the pTF7EMCV-1 vaccinia expression vector (16) and expressed in BS-C-1 cells with or without MT1-MMP using the infection-transfection procedure. For comparison, the cells were infected-transfected to co-express TIMP-2 and MT1-MMP. Analysis of TIMP-4 and TIMP-2 expression was monitored by immunoprecipitation of the inhibitors from the media of ^{35}S-labeled cells. As shown in Fig. 1, both TIMP-2 (Fig. 1, lane 2) and TIMP-4 (Fig. 1, lane 4) were detected in the media when expressed without MT1-MMP. In contrast, co-expression of the inhibitors with MT1-MMP

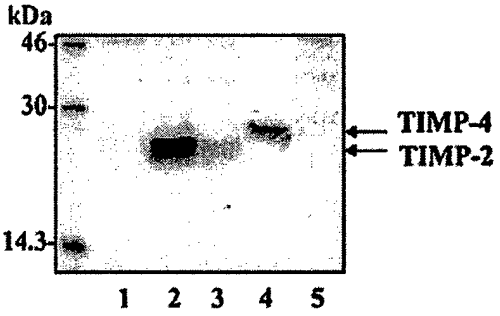

FIG. 1. Expression of TIMP-2 and TIMP-4 with and without MT1-MMP. BS-C-1 cells were infected–transfected to express TIMP-2 (lanes 2 and 3) or TIMP-4 (lanes 4 and 5) alone (lanes 2 and 4) or with MT1-MMP (lanes 3 and 5) as described under Materials and Methods. As control, cells were infected and then transfected with the empty vector. The next day, the media were collected and immunoprecipitated with antibodies to either TIMP-2 (lanes 2 and 3) or TIMP-4 (lanes 4 and 5) and Protein G-Sepharose beads. The medium of control-transfected cells (lane 1) was immunoprecipitated with both antibodies. The immunoprecipitates were resolved by reducing 15% SDS–PAGE followed by autoradiography. ^{14}C-labeled molecular weight standards were used as reference.

resulted in a significant reduction of TIMP-2 (Fig. 1, lane 3) and TIMP-4 (Fig. 1, lane 5) from the media. We have previously shown that co-expression of TIMP-2 with MT1-MMP results in the targeting of the inhibitor to the cell surface. This differential distribution is due to the binding of TIMP-2 to active MT1-MMP resulting in a specific downregulation of TIMP-2 protein from the media. Thus, the lack of detection of TIMP-4 in the supernatant of cells coexpressing MT1-MMP and TIMP-4 suggests that TIMP-4, like TIMP-2, associates with active MT1-MMP. Indeed, when coexpressed with MT1-MMP, TIMP-4 is found mostly in the cell lysate (shown in Fig. 3B, lane 3).

We next examined the ability of TIMP-4 to promote pro-MMP-2 activation by MT1-MMP. To this end, the BS-C-1 and the *Timp2* (−/−) mutant cells were infected-transfected to express MT1-MMP with or without TIMP-2 or TIMP-4. Then, the cells received exogenous pro-MMP-2. Activation was monitored by gelatin zymography of cell lysates. As shown in Fig. 2, BS-C-1 (Fig. 2A) or *Timp2* (−/−) mutant (Fig. 2B) cells coexpressing MT1-MMP and TIMP-2 (BS-C-1, Fig. 2A, lane 3; *Timp2* (−/−), Fig. 2B, lane 4) converted pro-MMP-2 (refer as P) into the active MMP-2 (refer as A) form of 62-kDa. In contrast, coexpression of MT1-MMP with TIMP-4 in either BS-C-1 (Fig. 2A, lane 4) or *Timp2* (−/−) mutant (Fig. 2B, lane 5) cells failed to promote pro-MMP-2 activation. BS-C-1 cells infected-transfected to express MT1-MMP alone (Fig. 2A, lane 2) exhibited a modest degree of active MMP-2 when compared to the activation observed when MT1-MMP was co-expressed with TIMP-2 (Fig. 2A, lane 3). This basal activation is due to the presence of some residual endogenous TIMP-2, which is present in the BS-C-1 cells, as reported previously (7). Indeed, in the *Timp2* (−/−) mutant cells, which are devoid of TIMP-2, no evidence of active MMP-2 was observed in cells expressing MT1-MMP alone (Fig. 2B, lane 3) consistent with the absolute requirement of TIMP-2 for the MT1-MMP-dependent activation of pro-MMP-2. As expected, no activation was observed in either cell type infected with the vTF7-3 virus and transfected with the empty vector (BS-C-1, Fig. 2A, lane 1; *Timp2* (−/−), Fig. 2B, lane 2). These studies establish the inability of TIMP-4 to promote pro-MMP-2 activation by MT1-MMP and demonstrate that this effect can only be mediated by TIMP-2 in spite of the ability of both inhibitors to form a non-covalent complex with pro-MMP-2 through its hemopexin-like domain (13, 15). The localization of TIMP-4 in the cell lysate when co-expressed with MT1-MMP suggested that TIMP-4 could bind to active MT1-MMP. Therefore, we wished to examine whether coexpression of TIMP-2 and TIMP-4 with MT1-MMP would affect the ability of TIMP-2 to enhance pro-MMP-2 activation. As shown in Fig. 2A, BS-C-1 cells coexpressing MT1-MMP, TIMP-2, and TIMP-4 (Fig. 2A, lane 5) exhibited a significant reduction in active MMP-2 when compared to the cells expressing MT1-MMP with TIMP-2 (Fig. 2A, lane 3). This result suggests that TIMP-4 competes for the binding of TIMP-2 to MT1-MMP and therefore prevents its ability to support pro-MMP-2 activation.

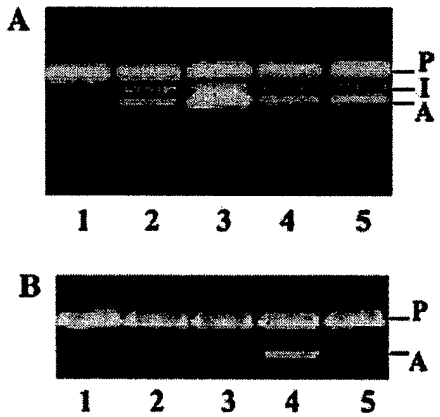

FIG. 2. Effect of TIMP-4 on pro-MMP-2 activation by MT1-MMP. (A) BS-C-1 cells were infected with 30 pfu/cell of vTF7-3 and then transfected with 0.4 μg/well each of plasmid DNA (lane 1, pTF7EMCV-1 empty vector; lane 2, pTF7-MT1; lane 3, pTF7-MT1 + pTF7-T2; lane 4, pTF7-MT1+ pTF7-T4; and lane 5, pTF7-MT1+ pTF7-T4 + pTF7-T2). Some cells were only infected with 30 pfu/cell of vTF7-3 (lane 1). At 18 h post-infection/transfection, the cells were incubated with 3 nM of purified recombinant pro-MMP-2 for an additional 4 h at 37°C followed by solubilization of the cells with lysis buffer. The lysates were then subjected to gelatin zymography. (B) *Timp2* (−/−) mutant cells were infected–transfected to express MT1-MMP alone (lane 3) or with either TIMP-2 (lane 4) or TIMP-4 (lane 5) as described in A. As control, some mutant cells were infected with vTF7-3 virus only or subsequently transfected with empty vector (lane 2). Activation of pro-MMP-2 was carried out as described in A. P, I, and A refer to pro-MMP-2 (72 kDa), intermediate form (64 kDa), and active MMP-2 (62 kDa).

Early studies showed a restricted pattern of expression of TIMP-4 in human and mouse tissues with preponderant expression in heart tissue (14, 23). However, recent studies indicate a broader tissue expression of TIMP-4 in normal tissues (24–26) and enhanced expression in pathological conditions (27–31). In various instances, the expression of TIMP-4 has been shown to overlap with that of TIMP-2 (27–29, 31). TIMP-4 has also been shown to inhibit tumor growth and metastasis of a human breast cancer cell line (32). However, its expression in human cancer tissues has not been examined in detail. Although the precise role of each TIMP in normal and pathological conditions remains to be further evaluated, the ability of TIMP-4 to inhibit the effect of TIMP-2 on pro-MMP-2 activation suggests that a balance of these inhibitors may alter the net activity of MMP-2 with TIMP-2, under certain conditions, promoting MMP-2- and MT1-MMP-dependent proteolysis and TIMP-4 acting as a general MMP inhibitor.

Presently, no information exists in regards to the molecular interactions of TIMP-4 with MT1-MMP. The crystal structures of TIMP-2 and TIMP-1 show that TIMP-2 has an insertion at Asp^{30}-Arg^{42} that extends the beta-sheets to interact with the catalytic domain of MT1-MMP. In addition, Tyr^{36} on TIMP-2 has hydrophobic interactions with the catalytic domain of MT1-MMP, fitting into a hydrophobic pocket above the active site groove near the S2/S3 site (5). These features, which are absent in TIMP-1, may partly explain the preferred binding of TIMP-2 to MT1-MMP (3, 5, 6). Energy-minimized computer modeling of the TIMP-4 structure based on the x-ray crystal structure of TIMP-2 (5) shows that TIMP-4 (12, 14), as opposed to TIMP-1, possesses a similar insertion at Val^{29}-Met^{41} that could potentially enhance interactions with the catalytic domain of MT1-MMP (Drs. Mobashery and Kotra, Dept. of Chemistry, Wayne State University, Personal communication) and inhibit MT1-MMP activity. We have previously shown that TIMP-2 induces accumulation of the 57-kDa active form of MT1-MMP (6). This effect is a direct consequence of the inhibitory action of TIMP-2 on MT1-MMP activity, which inhibits the autocatalytic turnover of active MT1-MMP into the inactive forms of 44–40 kDa. The 44-kDa form possesses an N-terminus starting at G^{285} and its formation involves the deletion of the entire catalytic domain of MT1-MMP (6). Accumulation of active MT1-MMP and reduction of the 44-kDa form are also observed with synthetic MMP inhibitors consistent with the autocatalytic nature of this process (6). Thus, both TIMP-2 and synthetic MMP inhibitors regulate the nature of the MT1-MMP forms present in the cells. Here, we examined the lysates of BS-C-1 cells expressing MT1-MMP alone or with TIMP-4 for the profile of MT1-MMP forms by immunoblot analysis. As shown in Fig. 3, in the absence of TIMP-4, MT1-MMP is detected in its 60-, 57-, and 44–40-kDa forms (Fig. 3, lane 2) as previously reported (6). The 60-kDa protein represents pro-MT1-MMP starting at S^{24} (6). The 62-, 55-, and 37–35 kDa bands are nonspecific, as they are detected in infected cells transfected with empty vector (Fig. 3, lane 1). In the presence of TIMP-4 (Fig. 3, lane 3), there is a significant increase in the 57-kDa species of MT1-MMP and a reduction in the 44-40-kDa forms. Figure 3B shows the presence of TIMP-4 in the same lysates as detected with a specific anti-TIMP-4 antibody (lane 3) (21). Similar results were obtained with the *Timp2* (−/−) mutant cells (data not shown). From these results we conclude that, like TIMP-2, TIMP-4 inhibits the autocatalytic turnover of MT1-MMP and thus, TIMP-4 is likely to form a complex with the active form of MT1-MMP. This is further supported by the lack of detection of TIMP-4 in the supernatant of cells co-expressing MT1-MMP with the inhibitor as discussed above. Although not directly proven here, these results suggest that the lack of pro-MMP-2 activation in the presence of TIMP-4 is due to the inability of this inhibitor to generate a ternary complex with MT1-MMP and pro-MMP-2. Thus, while TIMP-4 binds to active MT1-MMP, the TIMP-4/MT1-MMP complex cannot act as a receptor for pro-MMP-2 and therefore activation does not ensue. The structural constrains that impede the formation of the ternary complex awaits elucidation of the crystal structure of the TIMP-4/MT1-MMP complex. In conclusion, the results of this study demonstrate a differential role for members of the TIMP family in the inhibition of MT1-MMP activity and in MT1-MMP-dependent pro-MMP-2 activation.

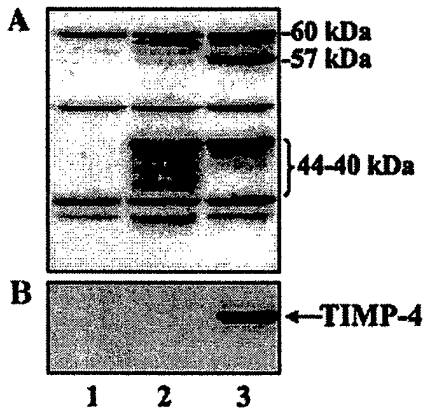

FIG. 3. Profile of MT1-MMP forms in the presence of TIMP-4. (A) BS-C-1 cells were infected–transfected to express MT1-MMP alone (lane 2) or with TIMP-4 (lane 3) as described in the legend to Fig. 2A. As control, some cells were infected–transfected with empty vector (lane 1). The cells lysates were resolved by reducing 10% SDS–PAGE followed by immunoblot analysis with anti-MT1-MMP antibodies and detection by ECL. (B) The same blot was reprobed with anti-TIMP-4 antibodies and developed by ECL.

AKNOWLEDGMENTS

Supported by National Institutes of Health (NIH) Grant CA-61986, Department of Defense (DOD) Grant DAMD17-99-1-9440 (both to R.F.), and NIH Grant CA-78646 (to Q-X.S.). S. H-B. was supported by a DOD Predoctoral Fellowship DAMD17-99-1-9441.

REFERENCES

1. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I. (1995) *J. Biol. Chem.* **270,** 5331–5338.
2. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. (1994) *Nature* **370,** 61–65.
3. Will, H., Atkinson, S. J., Butler, G. S., Smith, B., and Murphy, G. (1996) *J. Biol. Chem.* **271,** 17119–17123.
4. Butler, G. S., Butler, M. J., Atkinson, S. J., Will, H., Tamura, T., van Westrum, S. S., Crabbe, T., Clements, J., d'Ortho, M. P., and Murphy, G. (1998) *J. Biol. Chem.* **273,** 871–880.
5. Fernandez-Catalan, C., Bode, W., Huber, R., Turk, D., Calvete, J. J., Lichte, A., Tschesche, H., and Maskos, K. (1998) *EMBO J.* **17,** 5238–5248.
6. Hernandez-Barrantes, S., Toth, M., Bernardo, M. M., Yurkova, M., Gervasi, D. C., Raz, Y., Sang, Q. A., and Fridman, R. (2000) *J. Biol. Chem.* **275,** 12080–12089.
7. Toth, M., Bernardo, M. M., Gervasi, D. C., Soloway, P. D., Wang, Z., Bigg, H. F., Overall, C. M., DeClerck, Y. A., Tschesche, H., Cher, M. L., Brown, S., Mobashery, S., and Fridman, R. (2000) *J. Biol. Chem.* **275,** 41415–41423.
8. Zucker, S., Drews, M., Conner, C., Foda, H. D., DeClerck, Y. A., Langley, K. E., Bahou, W. F., Docherty, A. J., and Cao, J. (1998) *J. Biol. Chem.* **273,** 1216–1222.
9. Jo, Y., Yeon, J., Kim, H. J., and Lee, S. T. (2000) *Biochem. J.* **345** (Part 3), 511–519.
10. Hotary, K., Allen, E., Punturieri, A., Yana, I., and Weiss, S. J. (2000) *J. Cell. Biol.* **149,** 1309–1323.
11. Caterina, J. J., Yamada, S., Caterina, N. C., Longenecker, G., Holmback, K., Shi, J., Yermovsky, A. E., Engler, J. A., and Birkedal-Hansen, H. (2000) *J. Biol. Chem.* **275,** 26416–26422.
12. Brew, K., Dinakarpandian, D., and Nagase, H. (2000) *Biochim. Biophys. Acta* **1477,** 267–283.
13. Bigg, H. F., Shi, Y. E., Liu, Y. E., Steffensen, B., and Overall, C. M. (1997) *J. Biol. Chem.* **272,** 15496–154500.
14. Greene, J., Wang, M., Liu, Y. E., Raymond, L. A., Rosen, C., and Shi, Y. E. (1996) *J. Biol. Chem.* **271,** 30375–30380.
15. Fridman, R., Fuerst, T. R., Bird, R. E., Hoyhtya, M., Oelkuct, M., Kraus, S., Komarek, D., Liotta, L. A., Berman, M. L., and Stetler-Stevenson, W. G. (1992) *J. Biol. Chem.* **267,** 15398–15405.
16. Fuerst, T. R., Earl, P. L., and Moss, B. (1987) *Mol. Cell. Biol.* **7,** 2538–2544.
17. Soloway, P. D., Alexander, C. M., Werb, Z., and Jaenisch, R. (1996) *Oncogene* **13,** 2307–2314.
18. Wang, Z., Juttermann, R., and Soloway, P. D. (2000) *J. Biol. Chem.* **275,** 26411–26415.
19. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
20. Toth, M., Gervasi, D. C., and Fridman, R. (1997) *Cancer Res.* **57,** 3159–3167.
21. Li, H., Bauzon, D. E., Xu, X., Tschesche, H., Cao, J., and Sang, Q. A. (1998) *Mol. Carcinog.* **22,** 84–94.
22. Gervasi, D. C., Raz, A., Dehem, M., Yang, M., Kurkinen, M., and Fridman, R. (1996) *Biochem. Biophys. Res. Commun.* **228,** 530–538.
23. Leco, K. J., Apte, S. S., Taniguchi, G. T., Hawkes, S. P., Khokha, R., Schultz, G. A., and Edwards, D. R. (1997) *FEBS Lett.* **401,** 213–217.
24. Wu, I., and Moses, M. A. (1998) *Matrix Biol.* **16,** 339–342.
25. Riley, S. C., Leask, R., Denison, F. C., Wisely, K., Calder, A. A., and Howe, D. C. (1999) *J. Endocrinol.* **162,** 351–359.
26. Fata, J. E., Leco, K. J., Moorehead, R. A., Martin, D. C., and Khokha, R. (1999) *Dev. Biol.* **211,** 238–254.
27. Dollery, C. M., McEwan, J. R., Wang, M., Sang, Q. A., Liu, Y. E., and Shi, Y. E. (1999) *Circ. Res.* **84,** 498–504.
28. Michael, M., Babic, B., Khokha, R., Tsao, M., Ho, J., Pintilie, M., Leco, K., Chamberlain, D., and Shepherd, F. A. (1999) *J. Clin. Oncol.* **17,** 1802–1808.
29. Reno, C., Boykiw, R., Martinez, M. L., and Hart, D. A. (1998) *Biochem. Biophys. Res. Commun.* **252,** 757–763.
30. Thomas, P., Khokha, R., Shepherd, F. A., Feld, R., and Tsao, M. S. (2000) *J. Pathol.* **190,** 150–156.
31. Selman, M., Ruiz, V., Cabrera, S., Segura, L., Ramirez, R., Barrios, R., and Pardo, A. (2000) *Am. J. Physiol. Lung Cell Mol. Physiol.* **279,** L562–L574.
32. Wang, M., Liu, Y. E., Greene, J., Sheng, S., Fuchs, A., Rosen, E. M., and Shi, Y. E. (1997) *Oncogene* **14,** 2767–2774.